OPTIMIZING THE PRODUCTION OF BACTERIOCINS BY LACTIC ACID BACTERIA ISOLATED FROM FOODS USING IMPROVED DEFERRED ANTAGONISM ASSAY

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI'I IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

FOOD SCIENCE

DECEMBER 2017

By
Zhijun Zhan

Thesis Committee

Dr. Yong Li, Chairperson
Dr. Soojin Jun
Dr. Chin Nyean Lee

We certify that we have read this thesis and that, in our opinion, it is satisfactory in scope and quality as a thesis for the degree of Master of Science in Food science.

THESIS COMMITTEE

Chairperson:Dr.Yong Li

Dr. Soojin Jun

Dr. Chin Nyaan Lee

ACKNOWLEDGMENTS

I would acknowledge and express my deep gratitude to my thesis advisor, Dr. Yong Li, for his guidance, support and help with completing this research. The door to Dr. Li's office was always open whenever I ran into a trouble or had a question about my research or writing. Dr. Li has extraordinary patience, motivation, enthusiasm, and immense knowledge. With his guidance, I learned a lot, not only in the scientific area but also on a personal level. Guidance from Dr. Jin Dong was also critical to the completion of my research. She taught me about all the experimental skills during this study.

Besides my advisor, I would like to thank Dr. Soojin Jun and Dr. Chin Nyean Lee for their guidance in the creation of this thesis and for serving on my thesis committee. Thanks to Dr. Jun for his guidance in the courses that helped me obtain abundant knowledge about food processing and food engineering. His encouragement and support helped me stay active in food science research. Thanks to Dr. Lee who shared plentiful information about bacteriocins and probiotics with me. His help and guidance encouraged me to be confident in my research.

My sincere thanks go to my family for providing me with unfailing support and continuous encouragement throughout my two years of study and through the process of conducting research and writing this thesis. This accomplishment would not have been possible without them.

Finally, I would like to thank all my friends at University of Hawaii at Manoa for their help and support.

ABSTRACT

Bacteriocins are antimicrobial peptides or proteins produced by certain bacteria. Lactic acid bacteria (LAB) are common bacteriocin-producers and often used in the production of fermented food. These bacteria and bacteriocins they produce can inhibit certain bacteria causing food spoilage and foodborne disease. It is time consuming and labor intensive to isolate bacteriocin producing LAB from food. This study aimed to (1) improve the bacteriocin-producer isolation method, deferred antagonism assay, by optimizing media composition; (2) isolate and identify bacteriocin producing LAB from fermented foods; and (3) determine the influence of different growth conditions on the production of bacteriocins by those LAB isolates.

To identify more appropriate media to isolate bacteriocin-producers, three types of media (de Man, Rogosa and Sharpe [MRS] agar, M17 agar, and Elliker agar) with two types of buffering salts (disodium-β-glycerophosphate and the combination of Na_2HPO_4 and NaH_2PO_4) at different initial media pH (5.5-6.9) were tested with known six LAB strains via deferred antagonism assay. Tween 80 and ethanol were added at 1% to the isolation media to assess their effect on bacteriocin production. Both bacteriocin producing and bacteriocin non-producing LAB formed inhibition zones on MRS agar with two types of buffering salts. There was no inhibition zone formed by bacteriocin non-producers in the other two types of media (M17 agar and Elliker agar). The bacteriocin-producers generated significantly larger inhibition zones in Elliker agar than in M17 agar. The buffering salts did not significantly affect the size of inhibition zones. But disodium-β-glycerophosphate was known to inhibit the growth of *Lactobacillus bulgaricus* in previous study. The size of inhibition zones, formed by

the tested bacteriocin producing LAB, enlarged with the application of higher initial media pH and the supplement 1% Tween 80. Therefore, the Elliker agar with buffering salts (Na_2HPO_4 and NaH_2PO_4) and 1% Tween 80 at pH 6.9 was the most appropriate bottom media in deferred antagonism assay.

The improved deferred antagonism assay was employed to isolate bacteriocin producing LAB from kimchee, sauerkraut, yogurt and kefir. Bacteriocin-encoding genes in the isolates were amplified and sequenced. In addition, the inhibition spectrum of the isolates' bacteriocins was also determined by inoculated with four common pathogenic bacteria. A total of 10 bacteriocin producing LAB were isolated from those fermented food samples, which included 8 *Lactococcus lactis* and 2 *Lactobacillus plantarum*. Based on the result of random amplification of polymorphic DNA-polymerase chain reaction, the *Lactococcus lactis* isolates and *Lactobacillus plantarum* isolates could be divided into 4 groups and 1 group, respectively. Isolated *Lactococcus lactis* carried nisin Z and lactococcin 972 genes, and isolated *Lactobacillus plantarum* carried plantaricin S gene. Three types of the *Lactococcus lactis* isolates not only inhibited the growth of *Listeria monocytogenes* but also showed antimicrobial activity against *Staphylococcus aureus*.

To optimize the production of bacteriocins by those LAB isolates, the influence of different liquid culture media (MRS, M17 and Elliker broth) at different initial pH (5.5-6.9) with 1% Tween 80 and/or 1% ethanol on their antimicrobial activity was determined. The *Lactococcus lactis* isolates produced more bacteriocins in MRS broth than in M17 broth and Elliker broth. In comparison, the type of culture medium did not significantly affect bacteriocin production by the *Lactobacillus plantarum* isolates. Higher initial medium pH

increased the bacteriocin production. The bacteriocin units of all isolates at pH 6.9 were more than three-fold higher than at pH 5.5. As for the supplements, 1% Tween 80 effectively increased bacteriocin production by the *Lactococcus lactis* isolates, and 1% ethanol showed remarkable enhancement in bacteriocin production by the *Lactobacillus plantarum* isolates.

In summary, the optimized bottom media improves the deferred antagonism assay and provides a more effective approach to isolating bacteriocin-producers. The bacteriocin producing LAB isolates identified in this study can potentially be used as starter cultures in fermented foods to improve their quality and safety. Further work is needed to purify their bacteriocins and test them as natural antimicrobials in food preservation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Introduction

Microbes are abundant and ubiquitous in our living surroundings. From about 10,000 years ago, when human beings were still hunting foods, there were already problems caused by microbes including food spoilage and the diseases caused by pathogens. With the development of agriculture, food preservation has become more and more important. Although humans did not realize the existence of microorganisms, they had already preserved their foods via fermentation around 4,000 B.C. In 1676, Antonie van Leeuwenhoek utilized a crude microscope to observe some small living things, which were microbes. About 200 years later, Lazzaro Spallanzani showed that meat would not spoil when it was boiled in a sealed container. In 1862, Louis Pasteur designed the “swan-necked flask” experiment to prove the existence of microorganisms in nature and microorganisms being a cause of food spoilage. These discoveries eventually led to the invention of canning of foods.

Nowadays, it is well known that human lives are closely intertwined with microorganisms, which not only cause problems but also benefit humans. Diseases caused by foodborne pathogens are a major public health concern. According to the Centers for Disease Control and Prevention (CDC), the annual estimate of foodborne illness in the United States reaches 47.8 million, and 3,037 people die due to ingestion of contaminated food. To eliminate pathogens potentially present in food, a commonly used approach is pasteurization. However, consumers’ concern on the loss of nutrients during thermal processes has provoked increased demand for fresh and minimally processed foods. While these foods are usually

stored under refrigeration conditions, certain pathogenic bacteria, such as *Listeria monocytogenes*, have developed resistance to low temperature preservation and can grow in refrigerated foods.

Bacteriocins are antimicrobial proteins and peptides produced by bacteria. Lactic acid bacteria (LAB) are common bacteriocin-producers and widely found in fermented foods. The application of bacteriocin producing LAB as starter cultures in fermented foods can not only promote the fermentation by producing organic acids but also preserve the foods by inhibiting the growth of spoilage bacteria and pathogens. Bacteriocins produced by LAB have great potential as natural food preservatives. To isolate bacteriocin-producers from fermented foods, conventional methods are flip spot assay and flip streak method. These methods are very tedious and time consuming. Henning et al. (2015) designed a deferred antagonism assay which can simultaneously screen multiple LAB isolates for bacteriocin producing capability on agar directly. However, certain bacteriocin non-producing LAB can produce excessive organic acids or other antimicrobial compounds, which may interfere with the isolation of bacteriocin-producers in deferred antagonism assay.

The immediate objectives of this study were to improve deferred antagonism assay to reduce the chance of isolating bacteriocin non-producing LAB, and apply the improved method to isolate and identify bacteriocin producing LAB from certain fermented foods. Besides, the factors which can affect the production of bacteriocins by the isolates were investigated. The data from this study will hopefully facilitate the isolation of bacteriocin-producers and the discovery of novel bacteriocins which can be utilized in food preservation.

Chapter 2

Literature Review

2.1 Lactic acid bacteria

2.1.1 Introduction

Lactic acid bacteria (LAB) are characterized as Gram-positive, low-GC content, acid tolerant, usually non-motile and non-sporeforming bacteria, which produce lactic acid as a sole or major product of fermentative metabolism from glucose. The first pure culture of LAB was isolated by J. Lister in 1873, which was about ten years after Louis Pasteur's study on lactic acid fermentation (Shareck et al. 2004). The similarity between milk souring bacteria and other lactic acid-producing bacteria was recognized in the early 1900s. In 1890, cheese and sour milk were introduced; nowadays, LAB are commonly used as starter cultures in a variety of fermented foods, such as yogurt, kefir, butter milk, and kimchee.

According to cell morphology, members of LAB can be divided into rod and cocci. Lactobacilli and *Carnobacterium* are typical rod-shaped LAB. The other LAB genera are cocci. Besides, according to their glucose fermentation modes, LAB can be divided into homofermentative and heterofermentative bacteria. Homofermentative LAB convert sugars to lactic acid as their sole end product, which include *Lactococcus*, *Pediococcus*, and *Streptococcus* and so on. The role of homofermentative LAB in food fermentation processing is mainly related to decreasing the pH of food by producing enough lactic acids. In contrast to homofermentative LAB, heterofermentative LAB, like *Leuconostoc* and some *Lactobacillus* species, utilize the pentose phosphate pathway to replace the phosphoketolase pathway or phosphogluconate pathway, which can produce not only lactic acid but also acetic acid,

ethanol and/or carbon dioxide (König and Fröhlich, 2017). Therefore, heterofermentative LAB contribute to the texture and flavor of fermented foods.

2.1.2 Characteristics of major lactic acid bacteria

Due to ubiquitous occurrence of LAB in food and their significant contributions to human gut health, plenty of research has been focused on the characterization of LAB in hope of gaining better understanding of their physiology and exploring the potential of their metabolites as natural preservatives. The major genera of LAB are *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus*, and *Streptococcus*. The other LAB, including *Aerococcus, Carnobacterium, Enterococcus, Oenococcus, Sporolactobacillus, Tetragenococcus, Vagococcus*, and *Weissella*, belong to the order Lactobacillales.

2.1.2.1 Characteristics of *Lactobacillus*

Lactobacillus are gram-positive, facultatively anaerobic or microaerophilic, rod-shaped, non-sporeforming bacteria. The genomes of *Lactobacillus* are highly variable, which have 1,100 to 3,200 protein-coding genes (Mendes-Soares et al., 2014). The wealth of genome compositions attributes to high diversity among species in the genus *Lactobacillus*. Julius et al. (2008) isolated 23 representative *Lactobacillus* strains from fermented milk to test their functional characteristics. They reported that *Lactobacillus spp.* showed a high tolerance to acidic conditions of pH 2.5. *Lb. fermentum* strains had the highest resistance to acidic conditions, which could maintain 100% survival after exposure to pH 2.0. In addition, *Lactobacillus spp.* also showed bile toxicity resistance, antibiotic susceptibility, hydrophobicity, mucin degradation, DNase activity and antigenotoxic characteristics

(Guarner and Schaafsma, 1998).

2.1.2.2 Characteristics of *Leuconostoc*

Leuconostoc are gram-positive, facultatively anaerobic, non-sporeforming, ovoid cocci. Some strains of *Leuconostoc* show thermoduric capacity and can resist pasteurization (Martley and Crow,1993). *Leuconostoc* cells are able to survive for a long time in unfavorable surroundings. Hostile environmental conditions promote the formation of slime or glycocalyx on *Leuconostoc* cells, resulting in biofilms which protect the cells against detrimental agents (Kim et al., 2000). The slime formed by *Leuconostoc spp.* can also cause food products to spoil (Ennahar et al., 2003). In dairy technology, *Leuconostoc spp.* play an important role as non-starter lactic acid bacteria (NSLAB). As heterofermentative bacteria, *Leuconostoc spp.* contribute to the formation of aroma and texture of specific dairy products.

2.1.2.3 Characteristics of *Pediococcus*

Pediococcus are gram-positive, facultatively anaerobic, non-sporeforming, cocci. Pediococci are the major component of microbial flora in various types of crops (Cai et al., 1999). They commonly grow with other plant-associated LAB during fermentation. *Pediococcus* can utilize many carbohydrates as their carbon sources, which can ferment maltose, sucrose, and methyl glucoside to produce lactic acid as the sole end product (Bergan et al., 1984). In addition, *Pediococcus* can stand harsh environments. For example, *P. pentosaceus* shows tolerance to 1,0% NaCl. *P. pentosaceus* and *P. acidilactici* can survive at 50℃.

2.1.2.4 Characteristics of *Lactococcus*

Lactococcus are gram-positive, facultatively anaerobic or microaerophilic, non-sporeforming, non-motile, cocci. Lactococci are mesophilic and alkaline susceptive, which can grow at 10℃ but cannot be cultured at 45℃ or pH 9.6 (Cogan et al., 1997). Some *Lactococcus lactis* strains are tolerant to stressful conditions. Mannu et al. (2000) reported six strains of *L. lactis* were able to grow in medium containing 6.5% NaCl. *Lactococcus*, particularly *L. lactis*, are commonly used in the dairy industry to manufacture fermented foodssuch as cheeses. *L. lactis subsp. lactis* and *L. lactis subsp. cremoris* are important starter cultures in dairy fermentation (Hayes et al., 2006). Those strains can rapidly acidify the milk by fermenting lactose and glucose to produce lactic acid, which in turn inhibits the growth of spoilage bacteria. Additionally, those strains also contribute to the production of aroma compounds in fermented dairy products (Ayad et al. 1999). The flavor compounds produced by lactococci are due to their citrate fermentation, which can generate diacetyl, carbon dioxide, acetoin, pyruvate, 2,3-butanediol, and acetaldehyde (Bandell et al. 1998).

2.1.2.5 Characteristics of *Streptococcus*

Streptococcus are gram-positive, facultatively anaerobic, non-motile, non-sporeforming cocci. *Streptococcus* genus is common flora of the mouth, nose, and throat (Skinner and Quesnel 1978). Species of *Streptococcus* are classified based on their hemolytic properties (Patterson, 1996). Alpha-hemolytic species can oxidize the iron in hemoglobin molecules in red blood cells. Beta-hemolytic species can cause complete rupture of red blood cells. Gamma-hemolytic species cause no hemolysis. Some streptococci can cause human disease.

Strep. pyogenes, *Strep. agalactiae*, and *Strep. pneumonia* are notable as the pathogens of serious acute infections in human beings (Hardie and Whiley, 1995). Some *Strep. mitis* strains are aggressively pathogenic in immunologically compromised individuals, giving rise to septicemia or the adult respiratory distress syndrome (Hardie and Whiley, 1994). *Strep. anginosus* has been associated with abscesses in various parts of the body, such as the mouth, brain, liver and other organs (Whiley et al., 1992). Although some Streptococci can cause human diseases, many *Streptococcus* species are not pathogenic and can be applied in the food industry. Streptococci are critical ingredients in producing yogurts and Swiss cheeses by working as starter cultures (Beresford et al., 2001).

2.1.3 Metabolism of lactic acid bacteria

Kniel et al. (2012) mentioned in their book that carbohydrates are used as carbon and energy sources by homofermentative and heterofermentative LAB through different catabolic pathways, which are related to the formation of adenosine-tri-phosphate (ATP). There are three main catabolic pathways utilized by fermentive LAB, including Embden-Meyerhof-Parnas pathway, Enter-Doudoroff pathway, and Heterofermentative pathway. Figure 1 illustrates the simplified catabolic pathways of LAB.

The Embden-Meyerhof-Parnas (EMP) pathway is the most crucial catabolic pathway. In this pathway, LAB can convert one molecule of glucose to two molecules of pyruvic acids. However, at that point, the oxidation-reduction reactions are not balanced, which still need to oxidize the pyruvate to lactic acid. Meanwhile, two molecules of ATP generate. As the only end product of EMP pathway is lactic acid, EMP pathway is considered the most effective

pathway. The organisms using this pathway are called “homolactic”.

The Enter-Doudoroff pathway is more important in dairy fermentations than other two pathways. The reason is that milk contains abundant lactose which, as a disaccharide, can be broken down into glucose and galactose during fermentation. The other pathways cannot metabolize the galactose portion of lactose. Therefore, those LAB using EMP pathway or heterofermentative pathway will waste half of this energy source from lactose. However, those LAB which use Enter-Doudoroff pathway can metabolize galactose and produce one ATP molecule.

The LAB that utilize the heterofermentative pathway can metabolize five carbon sugars, such as pentoses. Hence, this type of LAB is more abundant in the environments that have adequate pentose and less hexoses.

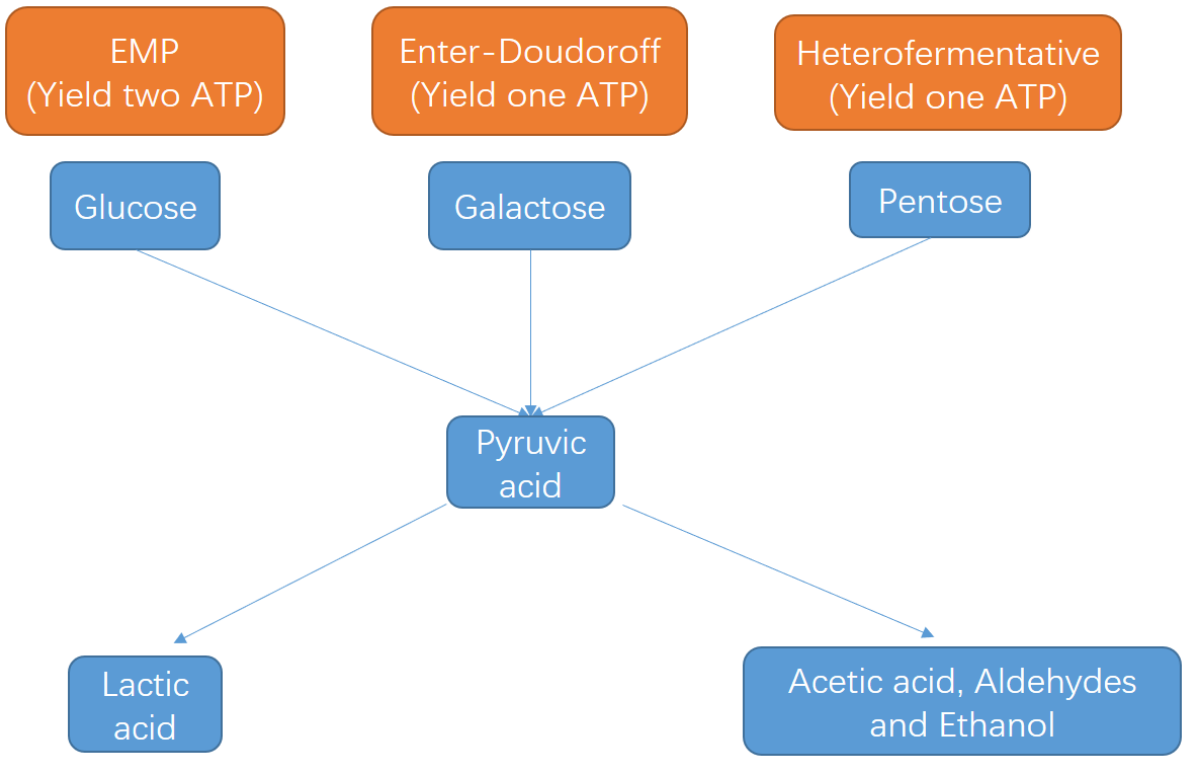


Figure 1. Simplified catabolic pathways of Fermentive LAB

2.1.4 Inhibition of LAB against spoilage bacteria and pathogens

The capacity of LAB to produce antimicrobial substances has been realized and used to preserve food since long time ago. Several investigations have proved that various species of LAB can exert antagonistic actions against intestinal and foodborne pathogens (Gibson et al., 1997). The antimicrobial activities of LAB may be due to a) decreasing pH by the production of organic acids; b) producing hydrogen peroxide; and c) producing bacteriocins (Sanders, 1993).

2.1.4.1 The effect of organic acids

LAB can produce and accumulate organic acids during food fermentation by metabolizing

carbohydrate source. The accumulation of lactic acid and short chain fatty acids (SCFA), such as acetic acid and propionic acid, results in a reduction in pH, which can inhibit many Gram-positive and Gram-negative bacteria. Lactic acid and acetic acid are known to inhibit *Staphylococcus aureus* in the early stage of its growth (Haines and Harmon, 1973). Goepfert and Hicks (1969) reported that *Salmonella* cells are inhibited when they grow at pH lower than 4.4. Adams and Hall (1988) showed that the combination of lactic acid and acetic acid can inhibit the growth of *E. coli* and *Salmonella*. The reason is that lactic acid can increase the mole ratio of inhibitory undissociated acetic acid. In addition, lactic acid and acetic acid can inhibit the growth of *Helicobacter pylori* that might cause ulcers and even stomach cancer (Midolo et al., 1995).

2.1.4.2 The effect of hydrogen peroxide

In aerobic conditions, LAB could produce hydrogen peroxide (H_2O_2). H_2O_2 can form destructive hydroxyl radical to peroxidate membrane lipids (Morris, 1979; Kong and Zotolla Zotolla Zotolla 1999on, 1980) and increase membrane permeability (Kong and Davison, 1980). Moreover, H_2O_2 can destruct nucleic acids and proteins in cell (Piard, and Desmazeaud, 1992). There are several reports on the effect of H_2O_2 produced by LAB on other microorganisms.

LAB can utilize the NADH oxidase, pyruvate oxidase, and NADH peroxidase to produce H_2O_2 (Murphy and Condon, 1984). H_2O_2-producing LAB are commonly present in the vagina of normal women, but they are absent from women with bacterial vaginosis. The production of H_2O_2 by LAB represents an antimicrobial defense mechanism of the normal vaginal ecosystem and protects against genital colonization by pathogens (Naidu et al., 1999). Hillier et al. (1992)

showed that women colonized by H_2O_2-positive LAB had lower chance to suffer bacterial vaginosis, symptomatic candidiasis, and vaginal colonization by *Gardnerella vaginalis*, *Bacteroides*, *Peptostreptococcus*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, and *Viridans streptococci*. Hawes et al. (1996) found that the women colonized by H_2O_2-producing LAB showed a decrease in the acquisition of vaginal infections.

2.1.4.3 The effect of bacteriocins

LAB can produce a wide range of bactericidal proteins which are deemed as bacteriocins. The production of bacteriocin is considered to be a strategy that certain bacteria use to compete with other bacteria. In recent years, there are an increasing number of studies on bacteriocins produced by LAB. For example, Nisin, as the most well-known bacteriocin, is produced by *Lactococcus lactis*, and can inhibit pathogenic *Listeria monocytogens*. Gibson et al. (1997) reported that *L. reuteri* produced the bacteriocin reuterin which inhibited *Salmonella* and *Listeria*. Jacobsen et al. (2003) utilized the living culture of *Leuconostoc carnosum* 4010 and its bacteriocin to effectively inhibit the growth of *Listeria monocytogenes*.More and more studies have focused on bacteriocins in that bacteriocins are considered natural and safe products to be applied in food systems, which are readily accepted by the consumer.

2.2 Bacteriocins

2.2.1 Introduction

Food spoilage and foodborne disease are common problems provoking concerns of food quality and public health all over the world. Bacterial pathogens, including *Salmonella enterica*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium botulinum*, and so on, have posed serious hazards to the public for many years. The infection by these pathogens can cause diarrhea, typhoidal fever, hemorrhage colitis, and even death. Many antibacterial substances produced by animals, plants, insects, and bacteria, such as hydrogen peroxide, fatty acids, organic acids, ethanol, antibiotics, and bacteriocins, have already been used by the food industry to improve the quality and safety of food products.

Bacteria can produce bacteriocins via their ribosomes as secondary metabolites. Bacteriocins are antibacterial peptides or proteins which are bactericidal or bacteriostatic. In comparison with chemical agents, bacteriocins are nearly harmless to humans because they lead to less modification of nutritional and organoleptic properties of foods and have less toxicity to human beings. Unlike antibiotics, bacteriocins cause less bacterial resistance, which indicates target bacteria might have further evolved. Bacteriocins are produced by bacteria to kill other related (narrow spectrum) or unrelated (wide spectrum) microbiota as their inherent defense weapons. They are a strategy for maintaining the population of bacteriocin-producers and reducing the number of competitors in hope of obtaining more nutrients and living space. LAB like *Lactococcus, Streptococcus, Pediococcus*, and *Lactobacillus* are major bacteriocin-producers and are commonly used in the production of

fermented foods, which reflects the safety of bacteriocins (Kniel et al., 2012). Therefore, it is necessary to understand the antimicrobial property of bacteriocins and isolate bacteriocin producing bacteria to find safer and more effective approaches to combating pathogens.

2.2.2 Classification

Both Gram-positive and Gram-negative bacteria can produce bacteriocins. However, the bacteriocin produced by Gram-positive species have boarder applications because their producers have already been used as starter cultures in fermented foods, which ensures the harmlessness of those bacteriocins. Those bacteriocins can be classified into four classes based on their molecular structure, molecular mass, thermostability and so on.

2.2.2.1 Class I-bacteriocin

Class I-bacteriocins represent those heat stable modified peptides containing unusual amino acids, such as lanthionine or methyllanthionine residues, which are called lantibiotics (Nissen and Nes, 1997). Based on the structure, this class can be subdivided into subclass Ia which includes relatively elongated, flexible, positively charged peptides, and subclass Ib which has globular, rigid and either negatively charged or uncharged peptides (Klaenhammer, 1993). LAB commonly produce this type of bacteriocin to attack other Gram-positive bacteria. Class I bacteriocins can bind to target molecules to prevent cell wall synthesis, and even get inserted into cell membranes and lead to the formation of pores on cell membranes causing cells to die.

2.2.2.2 Class-II bacteriocin

Class-II bacteriocins are those heat-stable, small and unmodified peptides, which do not contain any unusual amino acids, and are called non-lantibiotics. Class-II bacteriocins can be further divided into three subclasses (Ennahar et al., 2000). Subclass-IIa bacteriocins are mainly those pediocin-like antilisterial bacteriocins. Subclass-IIb bacteriocins have two peptides and are called two-component bacteriocins. Subclass-IIc bacteriocins are thiol-activated bacteriocins, such as circular bacteriocins. In general, Class II bacteriocin peptides can get into the membrane of target cells causing depolarization and death by applying their amphiphilic helical structures (Drider et al., 2006). The amphiphilic Class II bacteriocin peptides can combine with the N-terminal or C-terminal of target protein molecules and cause them to lose their activities.

2.2.2.3 Class-III bacteriocin

Class-III bacteriocins have apparent differences from the first two classes, which are heat-labile and larger (>10 kDa) protein molecules (Savadogo et al., 2006). Most of bacteriocins in this class are bacteriolysins which can lyse the target cell by cell wall hydrolytic activity (Johnsen et al., 2004). Lysostaphin produced by Gram-positive *Staphylococcus* species is a typical bacteriolysin, which can kill other Gram-positive bacterial cells through damaged cell walls (Cotter et al., 2005).

2.2.2.4 Class-IV bacteriocin

Those heat stable, complex bacteriocins, containing lipid or carbohydrate moieties,

belong to the Class-IV (Heng et al., 2007). This type of bacteriocins was found after the observation that bacteriocin activities had not been abolished until being treated with protease and glycolytic or lipolytic enzyme (Garneau et al., 2002). The mode action of this type of bacteriocins needs further study.

2.2.3 Difference between bacteriocins and antibiotics

Bacteriocins are antimicrobial peptides which have bactericidal or bacteriostatic mode of action against their producers' closely related species. Although the functions of bacteriocins and traditional antibiotics are similar, there are still remarkable differences between them. Bacteriocins have the potential to solve problems caused by the use of antibiotics and may take the place of antibiotics in foods and pharmaceuticals. Unlike antibiotics, bacteriocins are naturally produced by certain bacteria including the gut probiotic bacteria to against intestinal infections, which would not lead to collateral damage to the human commensal microbiota (Blaser, 2011). Those human commensal microbiotas play key roles in human health and are susceptive to antibiotics due to their broad-spectrum antimicrobial activities (Cotter et al., 2012). Moreover, bacteriocins are less toxic and have resulted in less incidence of atopic and autoimmune disease than antibiotics (Blaser, 2011). As to the development of cell tolerance to bacteriocin, any antimicrobial compounds including bacteriocin have the potential to cause resistance in microbiota. But some possible strategies can be used to minimize the emergence of bacteriocin resistance. For example, application of bacteriocins with distinct mechanisms of action in combination can keep their effects on the mutation of bacteria.

2.2.4 Factors affecting the production of bacteriocin

Bacteriocin biosynthesis occurs at the end of bacterial exponential growth phase (Piard and Desmazeaud, 1992). Bacteria produced bacteriocin to compete with other bacteria for getting more resources to grow. The production of bacteriocins by LAB can be affected by several factors, such as culture medium, culture pH, and medium supplement.

2.2.4.1 Effect of culture medium

Bacteriocin production is affected by the type of medium used to cultivate the bacteriocin-producers. The culture media which contain more essential nutrients required by bacteriocin-producers can promote their production of bacteriocins. Geis et al. (1983) compared the bacteriocin production of lactic streptococci grown in Elliker broth, M17 broth, and milk. The maximum bacteriocin production was found in Elliker broth followed by M17 broth. Piard et al. (1990) also observed that Elliker medium buffered with sodium β-glycerophosphate yielded more lacticin 481 than M17 medium. Muriana and Klaenhammer (1987) reported that the maximum lactacin F production was observed in MRS medium. Vignolo et al. (1995) reported that the production of lactocin 705 was much higher in MRS broth than in Elliker broth, M17 broth or BHI broth.

2.2.4.2 Effect of culture pH

Some LAB strains may prefer cultures with different pH and this can subsequently affect their production of bacteriocins. Cabo et al. (2001) found that the production of nisin by *Lactococcus lactis* was noticeably higher at pH 6.0 than at pH 5.5. Turgis et al. (2016)

showed that the production of nisin was significantly higher at pH 7 than pH 6.0 or pH 5.5. Vignolo et al. (1995) found that the maximum output of lactocin 705 by *Lactobacillus casei* CRL 705 was achieved in MRS broth at pH 6.5-7.5. *Lactobacillus sakei* subsp. *sakei* 2a secreted abundant bacteriocin at pH 5.5-7.0 (Malheiros et al., 2015). The production of lactococcin 140 was obtained at pH 5.5 (Parente et al., 1994). Mortvedt-Abildgaa (1995) reported that lactocin S would lose its bactericidal activity when the producer *Lactobacillus sake* L45 was cultured at pH higher than 6.0.

2.2.4.3 Effect of medium supplement

The addition of certain chemical reagents, such as tween and ethanol, into the culture medium may improve the production of bacteriocins by LAB. Tween (polysorbate) works as a surfactant which can increase the permeability of cell membranes and enhance the production of bacteriocins; it can also accelerate the diffusion of bacteriocins and improve their antimicrobial effect (Vignolo et al., 1995). Malheiros et al. (2015) reported that the addition of Tween 20 and Tween 80 increased the bacteriocin production by *L. sakei* 2a. Tween 20 can also enhance the activity of bacteriocins produced by *L. sakei* and *L. curvatus* ACU-1 (Castro MP. et al., 2011). Martinez et al. (2015) reported that Tween 80 can work as a stimulating additive to promote the production of bacteriocin-like inhibitory substances by *Bifidobacterium lactis* in skim milk. Radha and Padmavathi (2017) reported that 0.24% Tween 20 could increase the bacteriocin production by *Lactobacillus delbrueckii* subsp. *bulgaricus*. Ravi et al. (2017) observed that both Tween 20 and Tween 80 could increase the bacteriocin production by LAB isolated from mango pulp.

Additionally, low concentration of ethanol can cause slightly unfavorable growth condition which might increase the production of bacteriocin (De Vuyst et al., 1996). Mortvedt-Abildgaa et al. (1995) reported that low concentration of ethanol might prevent the bacteriocin aggregation and stabilize the bacteriocin. Callewaert et al. (1999) showed that ethanol not only stimulated the production of amylovorin L471 but also prevented the absorption of the bacteriocin to the producer cells during prolonged fermentation.

2.2.5 Application of Bacteriocin

Bacteriocins have many applications in food preservation, via directly adding into foods or in combination with other preservation approaches, such as pasteurization, high-pressure processing or irradiation (Abriouel et al., 2010; Keymanesh et al., 2009). The addition of pure or mixed bacteriocins or bacteriocin producing LAB to foods can increase their shelf life and safety by inhibiting common spoilage bacteria and foodborne pathogens. It can also control adventitious bacteria which may cause foreign odors. Bacteriocin producing LAB have been found in various fermented foods.

2.2.5.1 Application in dairy products

Many bacteria, particularly LAB, are used as starter cultures in fermented dairy products like yogurt, kefir, and cheese. Their antimicrobial products, bacteriocins, can even be utilized directly in those fermented dairy products. O'Sullivan et al. (2002) reported that bacteriocins were incorporated into cheese or yogurt as dried concentrate powders or used as the products of starter cultures. Many Swiss style cheeses rely on bacteriocin producing cultures to retard the gas blowing caused by clostridia. Nisin-producing Lactococci were found to effectively

act against clostridia spoilage (Hirsch et al., 1951). A combination of lactose fermenting, nisin-producing and proteinase-positive *L. lactis* strains showed antimicrobial effect on *Listeria monocytogenes*, *Clostridium sporogenes and Staphylococcus aureus* in cheese spreaders and pasteurized processed cheese (Zotolla 1994). Besides nisin, propionicin PLG-1 from *P. thoenii P127* is also commonly used in Swiss-type cheese. This bacteriocin can kill *Listeria monocytogenes, Pseudomonas fluorescens* and *Yersinia enterocolitica* (Lyon et al., 1993).

As for yogurt, applying a combination of nisin-producing strains and traditional starter cultures can prevent the growth of spoilage bacteria and extend its shelf life (Yamauchi *et al*., 1996). *Streptococcus thermophilus* is one of the starter cultures used in yogurt. It contributes to the development of yogurt semisolid texture by producing lactic acid. It also produces bacteriocins that can inhibit the growth of *Listeria monocytogenes* and to some extent, *S. aureus* (Yang et al., 2012). *Bifidobacterium* is common prebiotics used in yogurt, which can produce acidocin B. Brink et al. (1994) reported that acidocin B could act effectively against *Clostridium* sp. in fermented foods.

2.2.5.2 Application in meat products

For meat products, the contamination by *Listeria monocytogenes* is a serious concern. *L. monocytogenes* is widely distributed in nature and can get into ready-to-eat meat products (Nesbakken *et al*., 1996). Many bacteriocins produced by LAB showed their capability of reducing or inhibiting the growth of *L. monocytogenes* in meat products. Jacobsen *et al*. (2003) utilized the living culture of *Leuconostoc carnosum* 4010 and its bacteriocin in sliced

meat products. The treatment effectively inhibited the growth of *L.monocytogenes*. The bacteriocin sakacin P and pediocin AcH, isolated from *Lactobacillus* and *Pediococcus acidilactici* cultures, respectively, showed remarkable antilisterial activity in a *Listeria*-seeded raw pork meat matrix and suppressed *Listeria* for six weeks (Kouakou *et al*., 2010). Sabia et al. (2003) also reported that bacteriocin enterocin 416 K1 produced by *Enterococcus casseliflavus* showed strong antilisterial activity in Italian sausages.

In addition to those antilisterial bacteriocins, some other bacteriocins have also been isolated from fermented meat products, which can effectively treat and prevent microbial infections. For example, Todorov et al. (2010) found the bacteriocin producing strain *Enterococcus foecium* ST5Ha from smoked salmon can produce a pediocin-like bacteriocin. This bacteriocin inhibit not only *Listeria* spp. but also HSV-1 virus, which is a type of human virus.

2.2.5.3 Application in fruits and vegetables

Unlike meat products and dairy products, fruits and vegetables are consumed raw to keep their freshness. Unfortunately, some pathogenic microbes may contaminate those foods. A combination of bacteriocins and chemical preservatives can decrease the risk of microbial contamination. Molinos et al. (2008) reported that washing treatments with enterocin AS-48 could efficiently reduce the counts of *L. monocytogenes* in sliced melon, watermelon, pear, and kiwi in 24 hours. Moreover, a combination of enterocin AS-48 and 12 mM carvacrol, as well as with 100 mM n-propyl p-hydroxybenzoate showed increased antilisterial activity and suppressed the regrowth of *Listeria*. In addition to *Listeria*, enterocin AS-48 also had

inhibitor effect against *Staphylococcus aureus* in vegetable sauces. Although its antimicrobial activity was limited when it was used alone, a combination of AS-48 and 20 mM hydrocinnamic acid or 126 mM carvacrol could lower the viable count of *S. aureus* below the detection limit (Grande *et al*., 2007). Enterocin AS-48 also showed outstanding effect on the reduction of spoilage *Lactobacillus* strains, such as *Lb. collinoides* and *Lb. diolivorans,* when it was used along with high-intensity pulsed electric field treatment in apple juice (Martinez-Viedma et al., 2008).

There were other bacteriocins that can also be used to treat fresh fruits and vegetables. For example, Carvalho et al. (2008) reported remarkable effects of bovicin HC5, a bacteriocin produced by *S. bovis* HC5, against vegetative cells of *Alicyclobacillus acidoterrestris* which is a common spoilage bacterium in pasteurized acidic drinks. An interesting finding by Carvalho et al. was that spores of *A. acidoterrestris* were more sensitive to bovicin HC5 than vegetative cells.

2.2.6 Isolation of bacteriocin-producers

Approaches to isolating bacteriocin-producers are based on their antimicrobial capability. The first antimicrobial susceptibility test that utilized diffusion of the antibiotic substance through agar medium was done by Fleming in 1924 with penicillin against *Staphylococcus aureus* (Hoover and Steenson., 2014).

2.2.6.1 Flip streak method

This method requires the use of an aseptic loop to inoculate fresh LAB culture onto the surface of a culture medium agar by streaking. After incubation for 24 hours, the agar is

flipped onto the cover of the petri dish by using an aseptic spatula. Then the indicator strain is streaked on the inverted agar. After incubation for 24 hours, the bacteriocin producing strain can form inhibition line on the inverted plate (Spelhaug and Harlander, 1989). In this method, each plate usually just tests one type of LAB. Therefore, this method is complex and inefficient.

2.2.6.2 Flip spot method

Test organisms need to be cultured and diluted 10-fold in peptone water. Diluted cultures are spotted onto the surface of an agar plate. Each spot should keep approximately 3 cm distance. Plates are incubated for 24 hours, and the agar is reversed as in the flip streak method. The indicator strain is inoculated into temporarily melt soft agar and poured onto the surface of the inverted agar. This two-layers plate is incubated for 24 hours, and the inhibition capability of LAB is detected by observing inhibition zones (Spelhaug and Harlander, 1989). This method can identify multiple LAB strains at the same time. The disadvantage is that pure LAB cultures must be obtained before the inhibition test, which can be tedious and laborious.

2.2.6.3 Deferred antagonism assay

The sample containing mixed LAB strains is diluted and spread plated on the agar medium. A temporarily melt softer agar is poured onto the base agar immediately, and the two-layers plate was incubated for 24 hours. After the LAB colonies show up, a temporarily melt indicator layer with indicator organisms is overlaid on the two-layer plate. The three-layer plate is incubated for 24 hours until the indicator lawn grows to completion. The

bacteriocin producing LAB show their antimicrobial capability by forming clear inhibition zones (Henning et al., 2015). This method does not require the use of pure LAB cultures and can screen more LAB strains at one time than flip spot method. However, certain bacteriocin non-producers can produce excessive organic acids and also form inhibition zones in this assay.

Chapter 3

Improved deferred antagonism assay for isolating bacteriocin producing lactic acid bacteria

3.1 Introduction

Microbes play an important role in food safety. Pathogenic organisms can cause illness through contaminated food. According to Centers for Disease Control and Prevention (CDC), *Listeria monocytogenes* is one of the most dangerous foodborne pathogens, and it causes approximately 1,600 people to become sick and 260 people to die annually in the United States. *Listeria monocytogenes* is non-sporeforming, gram-positive, facultative anaerobic, rod shape bacterium. Although there is no evidence on the infectious dose of *Listeria monocytogenes* in humans, Golnazarian et al. (1989) reported that mice infected orally showed variable responses, with 50% infectious doses ranging from 1.7×10^3 to 9.9×10^6 CFU. *Listeria monocytogenes* infections can cause diarrhea, fever, septicemia, meningitis, encephalitis or abortion. Moreover, this pathogen has high resistance to undesirable environments, which can grow at 14% NaCl, pH 4.4, or - 0.1°C conditions (Walker et al. 1990). Therefore, those heat sensitive foods, such as fresh produce and fermented foods, are likely to harbor *Listeria* strains. To solve this problem, bacteriocin producing lactic acid bacteria (LAB) offer a natural and effective approach of eliminating or inhibiting the growth of *Listeria monocytogenes* in food.

LAB are gram-positive, acid tolerant, non-motile and non-sporeforming bacteria. LAB are widely used as starter cultures in fermented foods to increase their shelf life and quality. During the application of LAB in fermentation, their effect against other microbes have been

gradually realized by human beings. It is by now well known that LAB can produce antimicrobial compounds including organic acids, hydrogen peroxide, diacetyl, and bacteriocins.

Bacteriocins are bactericidal or bacteriostatic proteins produced by bacteria. Generally, LAB are deemed safe, and so are their bacteriocins, which should not negatively affect human health (Eijsink et al., 2002). Therefore, bacteriocins produced by LAB have attracted much attention. These bacteriocins can be separated into different classes and show different inhibitory spectra. Commonly, bacteriocins produced by LAB can be divided into two categories, lantibiotics and non-lantibiotics. Lantibiotics have post-translationally modified amino acids, such as dehydroalanine (Dha), dehydrobutyrine (Dhb), eponymous lanthionine (Lan), and β-methyllanthionine (MeLan) formed by thioether linkages between dehydrated amino acid residues and neighboring cysteines (Rink et al., 2007). Lantibiotics can prevent cell wall synthesis and form pores on cell membranes, which eventually cause the death of target cells. Non-lantibiotics do not have unusual amino acids. They can cause depolarization of target cells and even lyse their cell walls.

To isolate bacteriocin producing LAB, flip streak method and flip spot method are commonly used. These two methods require picking an excessive number of isolates from food samples followed by culturing them individually in broth, neutralizing organic acids by alkaline, and finally testing them on agar against indicator organisms. Therefore, both methods are time-consuming and pretty tedious to use. Yet the isolation rate can be low since most LAB cannot produce bacteriocins. Thus, a more efficient approach, bacteriocin deferred antagonism assay, was developed by Henning et al. (2015). This assay can test the bacteriocin

producing capability of more than 80 colonies on agar directly at the same time, which significantly reduce testing time and labor (Figure 2). However, the deferred antagonism assay still has limitations. LAB can produce lactic acids and acetic acid which can reduce the pH of media to around 4.0. At that pH, indicator bacteria like *Listeria monocytogenes* cannnot grow. Hence, there is a high possibility to isolate bacteriocin non-producing LAB which can inhibit the growth of *Listeria monocytogenes* by causing acidic surroundings in the deferred antagonism assay. To solve this problem, it was necessary to decrease the effect of acids produced by LAB on indicator bacteria while increasing the production of bacteriocins by LAB in the deferred antagonism assay.

The objectives of this study were to determine the effects of various agar media combined with different buffering salts on bacteriocin-producers and non-producers in deferred antagonism assay. So that more appropriate agar medium can be determined to rapid and effective isolation of bacteriocin producing LAB from foods. In addition, as LAB are sensitive to environmental acidity in producing bacteriocins, different pH of selected agar medium was tested separately in order to find the initial medium pH, which can ensure the production of bacteriocins by LAB and alleviate the interference of organic acids produced by bacteriocin nonproducing LAB. Finally, previous studies indicate that adding 1% Tween 80 to culture media can increase the production of lactocin 705 (Vignolo et al., 1995). Supplementation of culture media with 1% of ethanol can prevent lactocin S from aggregating and increase its activity (Mortvedt-Abildgaa et al., 1995). Therefore, both Tween 80 and ethanol were tested with selected agar medium for LAB in deferred antagonism assay.

3.2 Materials and Methods

3.2.1 Bacterial strains and growth conditions

Three previously identified bacteriocin producing *Lactococcus lactis* strains (Ki, L7-3, B) were used as bacteriocin positive control strains. Three bacteriocin non-producers, *Leuconostoc mesenteroides* (R7-4), *Leuconostoc pseudomesenteroides* (LPM) and *Lactobacillus plantarum* (LBP), were used as bacteriocin negative control strains. All these strains were grown in MRS (De Man, Rogosa and Sharpe) broth at 30℃ for 24 hours before being plated on agar media in deferred antagonism assay. The indicator organism, *Listeria monocytogene*, was grown in TSB (Tryptic soy broth) at 35℃. All strains were maintained as frozen stock cultures at -80℃ and propagated twice before being used in this study.

3.2.2 Bacteriocin deferred antagonism assay

Bacteriocin positive and negative control strains were serially diluted by 10-fold in 0.1% peptone water, and appropriate dilutions (250-2500 CFU/mL) were chosen to use in deferred antagonism assay. Figure 2 illustrates the deferred antagonism assay described by Henning et al. (2015), which spread all tested strains onto buffered base agar layer (1.5% agar). Plated samples were immediately covered with same buffered agar sandwich layer (0.75% agar). The sandwich layer was included to decrease the interference of lactic acid produced by LAB and prevent the inhibition zone caused by bacteria phage when dealing with food samples. After that, tested organisms in double-layer agar were incubated at 30℃ for 24 to 48h. The base layer and sandwich layer called as bottom media. When colonies could be observed in bottom media, 10 mL of molten BHI agar (0.75% agar) and 0.1 mL of 1×10^8 CFU/mL

Listeria monocytogenes were mixed and overlaid onto the sandwich layer. Finally, the agar plates were incubated at 30℃ until the indicator lawn formed. The bacteriocin producing strains can form inhibition zone around the colony as shown in Figure 2.

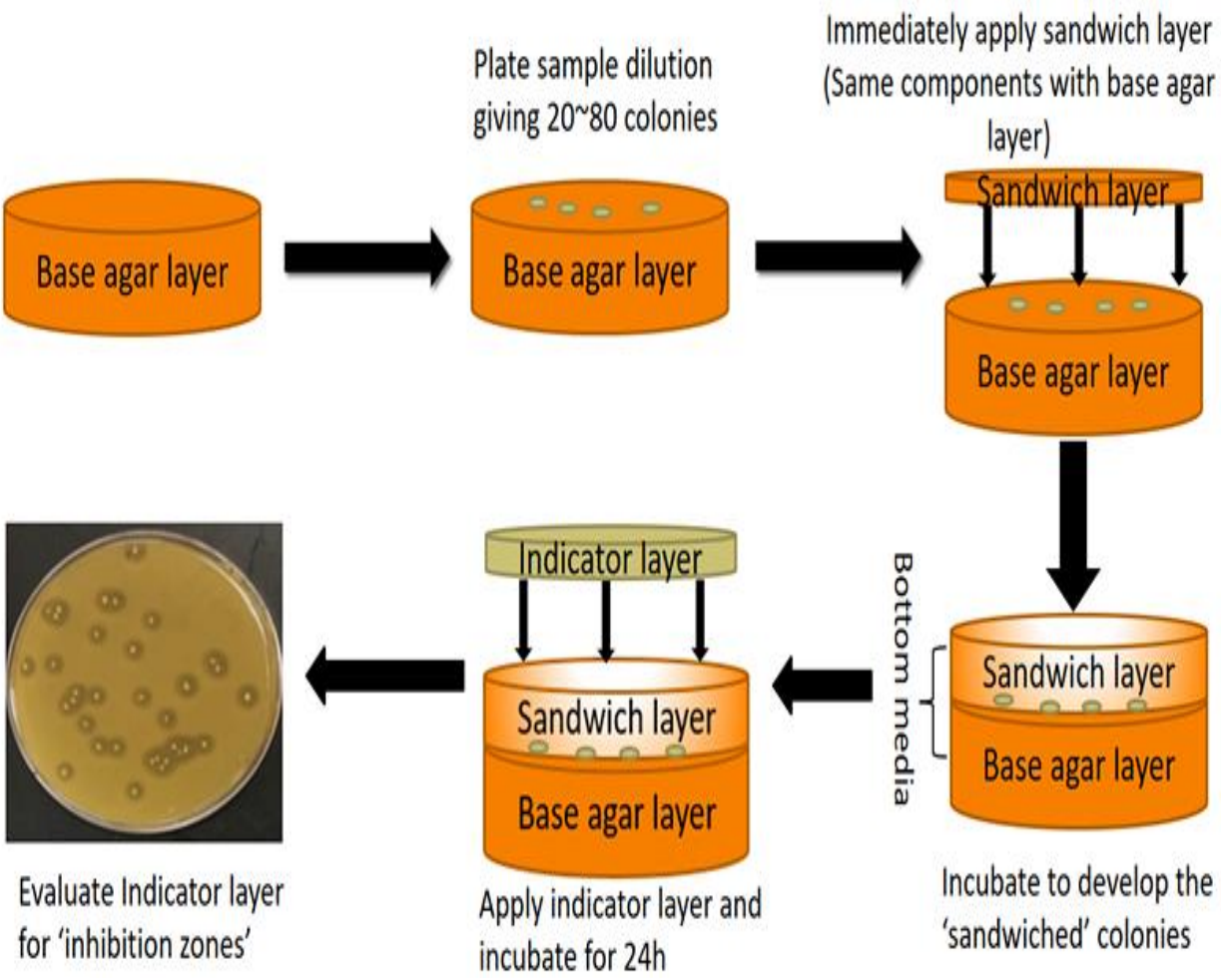


Figure 2. Bacteriocin deferred antagonism assay

3.2.3 Test different media in deferred antagonism assay

Three different agar were used as the bottom media to decide which type was more appropriate in deferred antagonism assay. They were MRS medium, M17 medium, and Elliker medium, which were suitable to grow most LAB species. In addition, two types of buffering salts were added into the agar media in order to alleviate interference of acids in bacteriocin detection. They were 2% β-glycerophosphate and the combination of sodium phosphate monobasic and dibasic (1% Na_2HPO_4 and 0.35% NaH_2PO_4) which gave the same medium pH (6.9±0.2). Table 1 illustrates the components of tested bottom media. The diameters of inhibition zones from different bottom media were recorded. The diameter of inhibition zone was the distance from the edge of a bacterial colony to the edge of surrounding clear zone.

Table 1. The components of tested bottom media

Bottom media	Buffering salt	
MRS	β-glycerophosphate	Na_2HPO_4 and NaH_2PO_4
M17	β-glycerophosphate	Na_2HPO_4 and NaH_2PO_4
Elliker medium	β-glycerophosphate	Na_2HPO_4 and NaH_2PO_4

3.2.4 Test different initial pH of the bottom media

LAB are sensitive to pH in producing bacteriocins. Commonly, LAB can produce bacteriocins in pH ranging from 5.5 to 7. Therefore, the three bacteriocin-producers and three bacteriocin non-producers were inoculate onto selected bottom media with initial pH 5.5, 6.0, 6.5 or 6.9. Then, the deferred antagonism assay was conducted as described above. The diameters of inhibition zones from bottom media with different pH were recorded.

3.2.5 Test the effect of Tween 80 and ethanol in bottom media

After an appropriate combination of the bottom media and buffering salts as well as the medium pH were determined, 1% Tween 80 and 1% ethanol were added individually and in combination to both base agar layer and sandwich layer to form new bottom media. Tween 80 and ethanol were filter-sterilized before being added to bottom media. The six tested strains were inoculated on base agar to check for the formation of inhibition zones by using the deferred antagonism assay. The diameters of inhibition zones from bottom media with or without Tween 80 and/or ethanol were recorded and compared.

3.2.6 Statistical analysis

All tests were repeated once. Four inhibition zones were selected from each strain and measured for the diameter. The inhibition zone sizes from different treatments were compared via ANOVA with a significance level of 0.05.

3.3 Results

3.3.1 Effect of bottom media agar type

To choose more appropriate agar as bottom media in the deferred antagonism assay, three bacteriocin-producers (Ki, L7-3 and B) and three bacteriocin non-producers (R7-4, LPM and LBP) were inoculated into three types of media with two types of buffering salts. Figure 3 illustrates the diameter of inhibition zones showed in different media. Figure 4 shows the effect of representative bacteriocin-producers and non-producers on the growth of *Listeria monocytogenes*. All strains including three bacteriocin non-producers, *Leuconostoc mesenteroides* (R7-4), *Leuconostoc pseudomesenteroides* (LPM) and *Lactobacillus plantarum* (LBP), formed inhibition zones in MRS agar with both buffering salts in deferred antagonism assay. The inhibition zones caused by bacteriocin non-producers were smaller than the zones caused by bacteriocin-producers. Bacteriocin non-producers did not show inhibition zones in M17 agar or Elliker agar. In comparison to M17 agar, Elliker agar was superior in deferred antagonism assay because bacteriocin-producers *Lactococcus lactis* L7-3 and *Lactococcus lactis* B showed larger diameters of inhibition zones when Elliker agar was used as the bottom media. This suggests Elliker medium might promote the production or the diffusion of bacteriocins. Therefore, Elliker medium was more appropriate than M17 medium to be used as the bottom media. Moreover, there was no significant difference between the two buffering salts in deferred antagonism assay. However, Shankar and Davies (1977) reported that β-glycerophosphate inhibited the growth of many *Lactobacillus* strains, particularly, *Lactobacillus bulgaricus*. Meanwhile, certain *Lactobacillus bulgaricus* strains can produce bacteriocins (Radha and Padmavathi, 2017). Hence, Elliker medium with the

combination of sodium phosphate dibasic and monobasic was considered more appropriate bottom media in deferred antagonism assay.

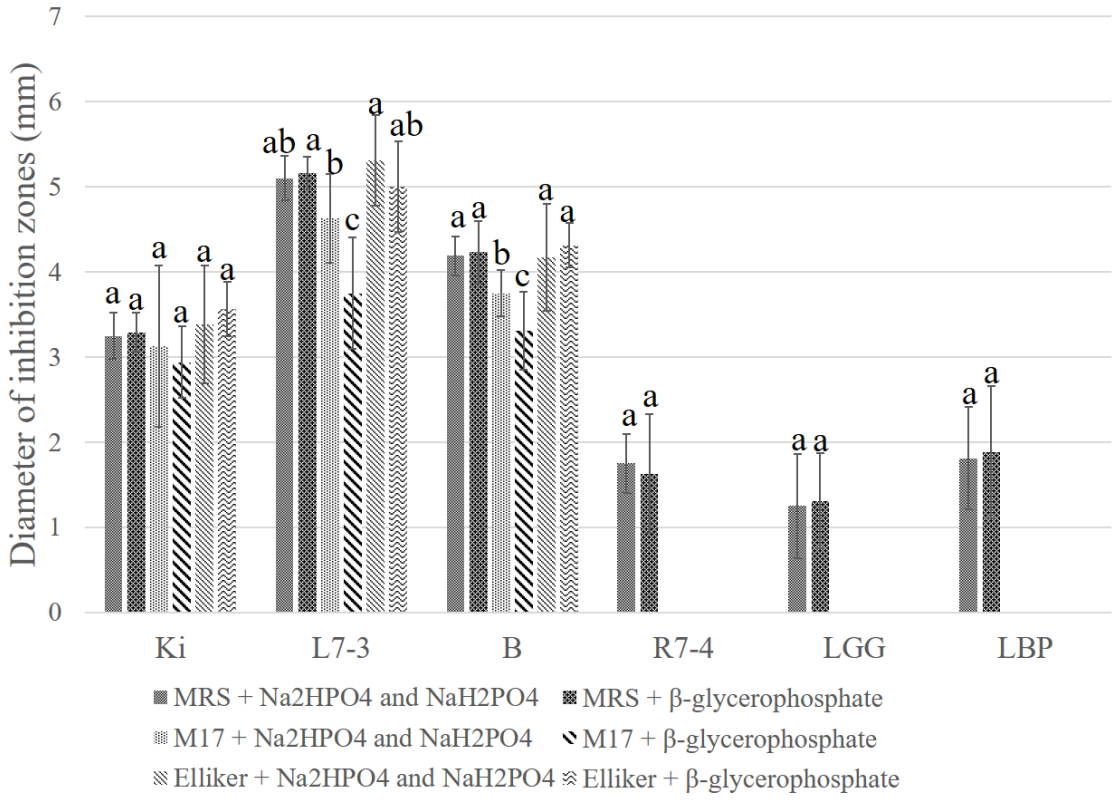


Figure 3. The effects of bottom media composition on the diameter of inhibition zones

Figure 4. The inhibition zones formed by representative bacteriocin-producers and bacteriocin non-producers in different media. Bacteriocin-producers (A, C, E, G, I, K) and Bacteriocin non-producers (B, D, F, H, J, L) on MRS (A, B, C, D), M17 (E, F, G, H) and Elliker medium (I, J, K, L) with buffering salts Na_2HPO_4 and NaH_2PO_4 (A, B, E, F, I, J) or β-glycerophosphate (C, D, G, H, K, L)

3.3.2 Effect of initial pH of bottom media

Elliker medium with the combination of sodium phosphate dibasic and monobasic was used as bottom media. The pH of bottom media was adjusted to 5.5, 6, 6.5 and 6.9, respectively. Their effects on the diameter of inhibition zone are shown in Figure 5. Those bacteriocin producing strains showed inhibition zones in the bottom media from pH 5.5 to 6.9. However, bacteriocin non-producing strains could also form inhibition zones at pH 5.5., which indicates that the bottom media at pH 5.5 should not be used in deferred antagonism assay. The diameter of inhibition zones created by the three bacteriocin producing *Lactococcus lactis* strains increased when the pH of bottom media rose. Figure 6 shows the inhibition zones formed by bacteriocin-producers and bacteriocin non-producers in the bottom media from pH 5.5 to 6.9. The pH value 6.9 was chosen for the bottom media in deferred antagonism assay.

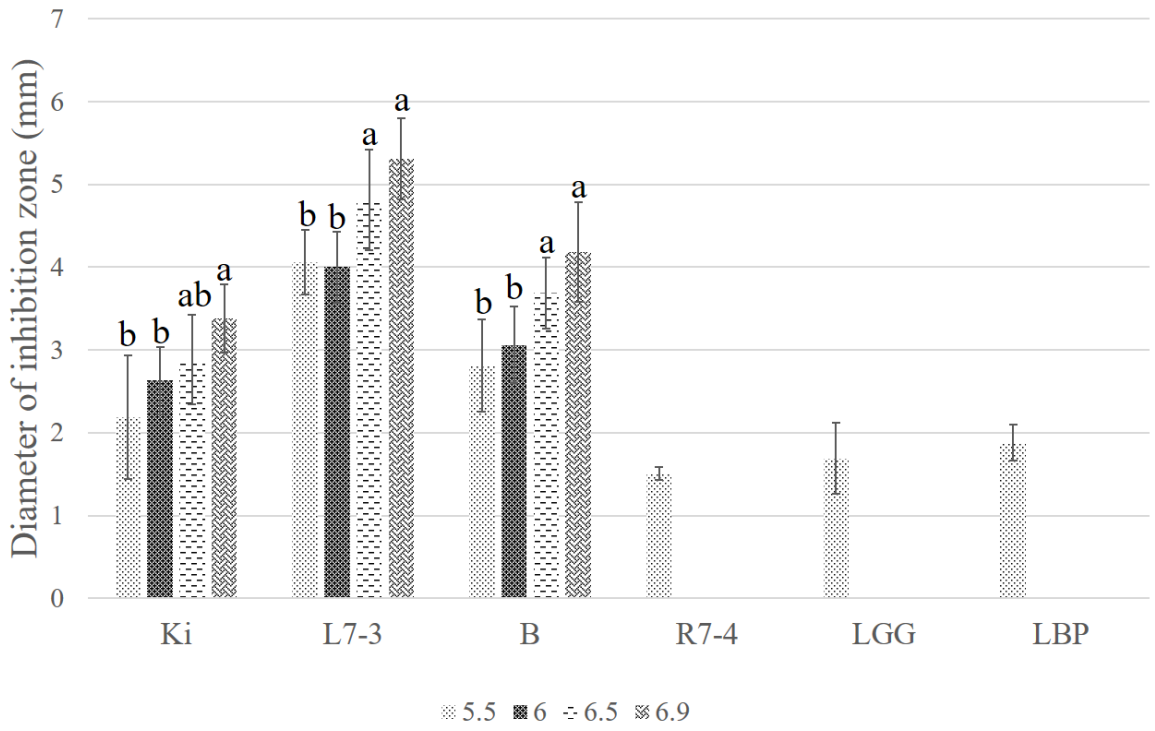


Figure 5. The effects of pH of the bottom media on the diameter of inhibition zones

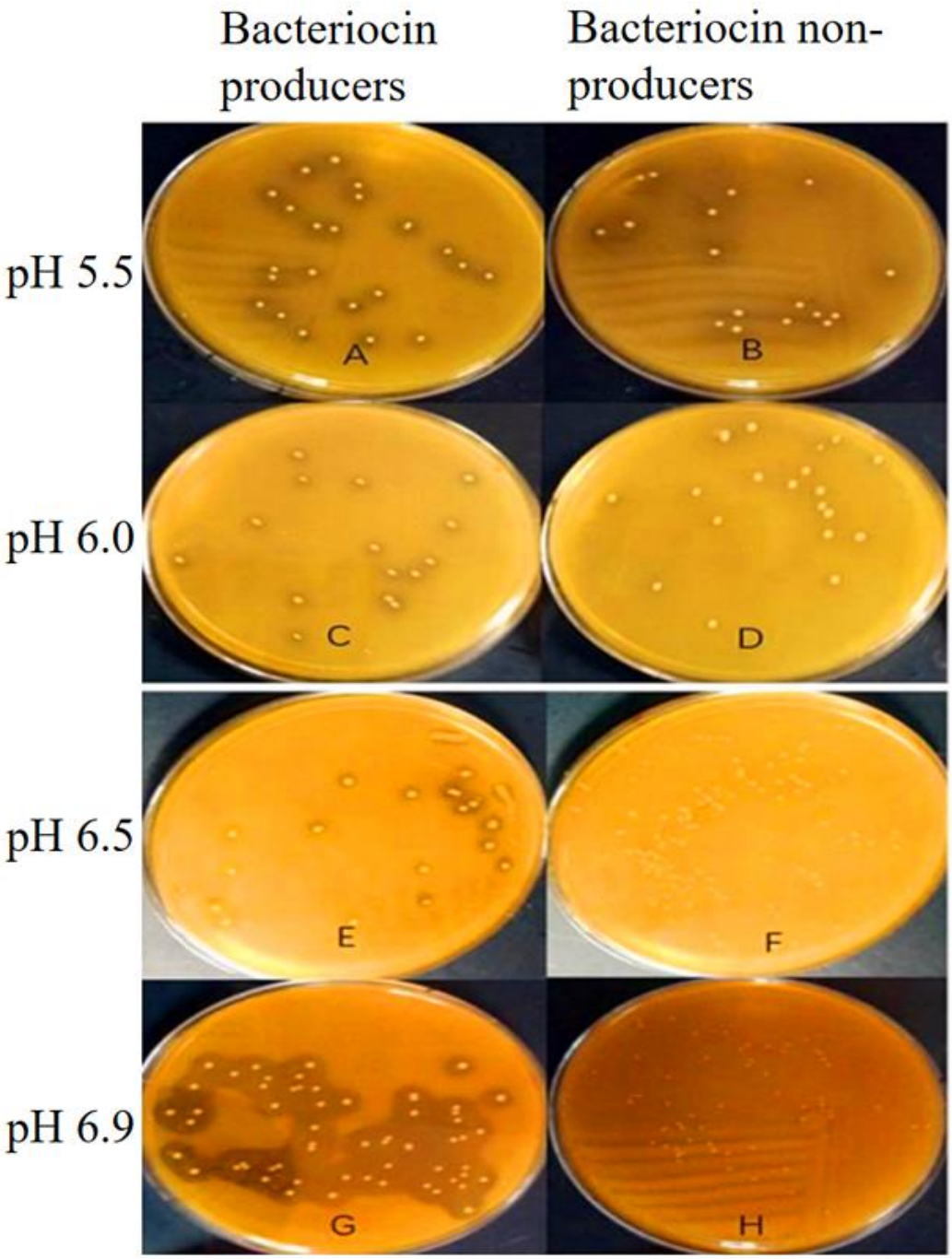


Figure 6. The inhibition zones formed by representative bacteriocin-producers and bacteriocin non-producers at different initial medium pH. Bacteriocin-producers (A, C, E, G) and bacteriocin non-producers (B, D, F, H) on Elliker medium at pH 5.5 (A, B), 6.0 (C, D), 6.5 (E, F) or 6.9 (G, H).

3.3.3 Effect of Tween 80 and ethanol added to the bottom media

1% Tween 80, 1% ethanol, and their combination were added separately to the bottom media at pH 6.9. The bottom media without these supplements was used as negative control. The effects of Tween 80 and ethanol on the diameter of inhibition zones are shown in Figure 7. No inhibition zones were formed by the three bacteriocin non-producers with the addition of the two supplements. For those three bacteriocin-producers, the ethanol treatment did not show significant effect on the diameter of inhibition zones. However, the addition of 1% Tween 80 led significantly increase in the diameter of inhibition zones. Besides, no significant difference was observed between the addition of 1% Tween 80 and the combination of 1% Tween 80 and 1% ethanol. Figure 8 illustrates the inhibition zones formed by representative bacteriocin-producers and bacteriocin non-producers on Elliker medium with 1% Tween 80, 1% ethanol, or the combination of 1% Tween 80 and 1% ethanol. Therefore, 1% Tween 80 should be chosen as a supplement to the bottom media in deferred antagonism assay.

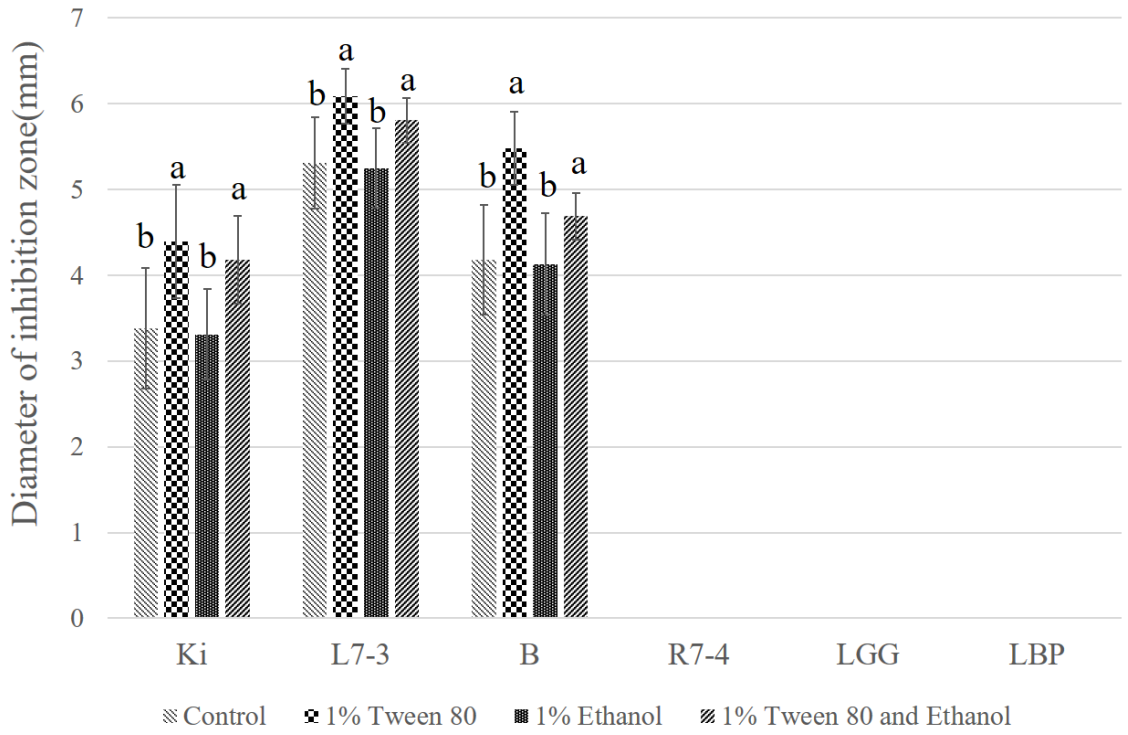


Figure 7. The effects of Tween 80 and/or ethanol on the diameter of inhibition zones

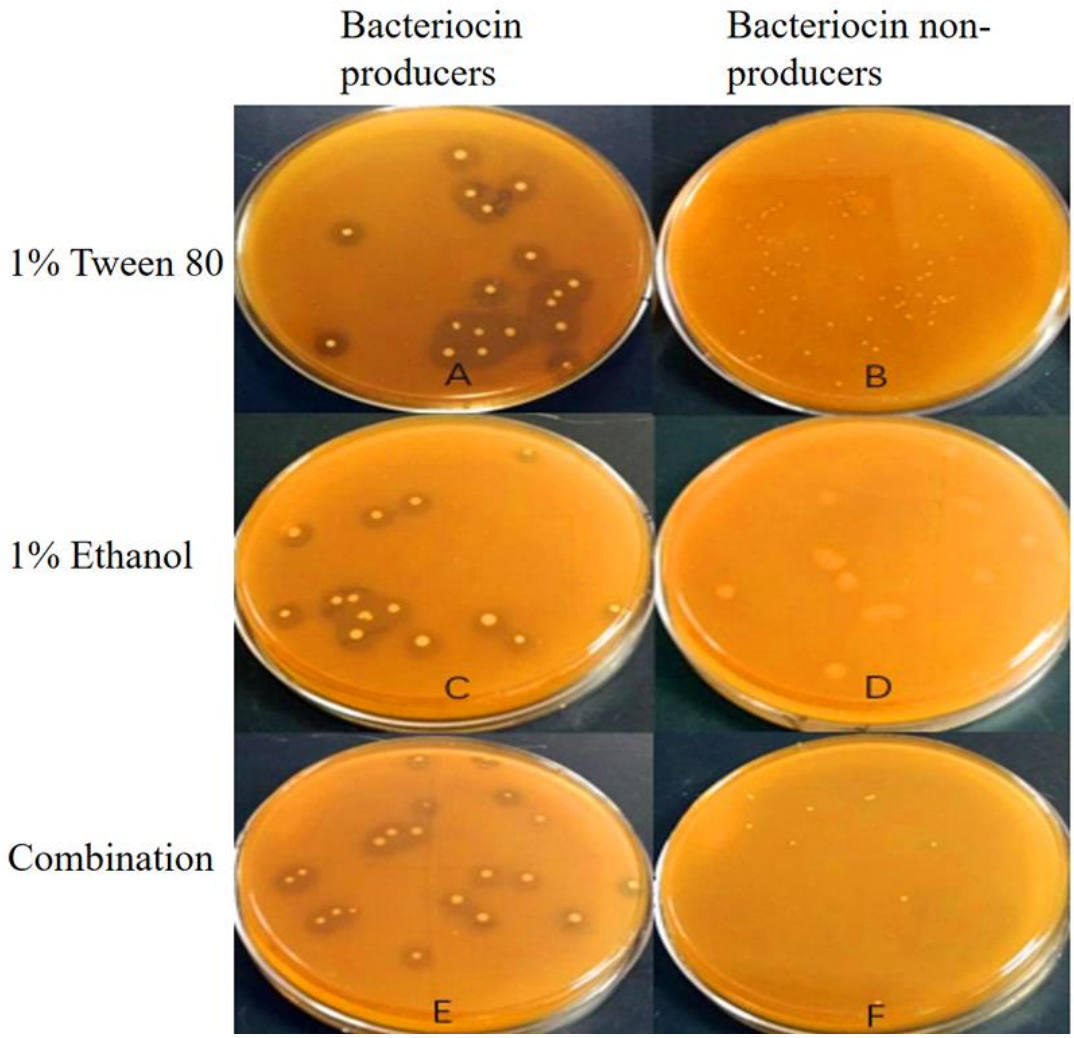


Figure 8. The of inhibition zones formed by representative bacteriocin-producers in the media with different supplements. Bacteriocin-producers (A, C, E) and Bacteriocin non-producers (B, D, F) on Elliker medium with 1% Tween 80 (A, B), 1% ethanol (C, D), or the combination of 1% Tween 80 and 1% ethanol (E, F)

3.4 Discussions

These results demonstrated the effects of the type and initial pH of bottom media, Tween 80, and ethanol on the diameter of inhibition zones caused by bacteriocin-producers in the deferred antagonism assay. The phenomenon that inhibition zones could be formed by bacteriocin non-producers when MRS agar was used as the bottom media was probably one of main reasons for the isolation of many non-specific inhibition LAB in deferred antagonism assay. MRS medium might promote the production of non-bacteriocin antimicrobial substances, like lactic acid. Naveena et al. (2005) reported that triammonium citrate, an ingredient only used in MRS agar but not the other two tested media, can significantly increase the production of lactic acid by *Lactobacillus casei*. This kind of substance can interfere with the detection of bacteriocin-producers. Although Vignolo et al., (1995) reported that MRS medium caused higher bacteriocin production than Elliker medium and M17 medium, this was not the case with bacteriocin producing *Lactococcus lactis* strains in this test. In deferred antagonism assay, bacteriocins need to diffuse through sandwich layer to inhibit the growth of *Listeria monocytogenes* in indicator layer, which might decrease the sensitivity of indicator organisms to bacteriocins. Elliker medium was more suitable for those bacteriocin producing *Lactococcus lactis* to produce bacteriocins, which yielded larger inhibition zones than M17 medium in deferred antagonism assay. Vignolo et al. (1995) and Geis et al. (1983) reported some bacteriocin producing LAB showed low bacteriocin activity after they grew in M17 medium. Therefore, M17 medium might interfere with the synthesis of bacteriocins by those LAB.

When Elliker agar and M17 agar were separately used as bottom media, both β-glycerophosphate and the combination of sodium phosphate monobasic and dibasic were able

to eliminate the interference of acids in deferred antagonism assay. Overall, the two buffering salts did not significantly affect the diameter of inhibition zones, indicating that they have similar buffering capacity. However, Shankar and Davies (1977) reported that 0.15% β-glycerophosphate could inhibited the growth of *Lactobacillus bulgaricus*; and many *Lactobacillus* strains cannot grow in 1.5% β-glycerophosphate. Considering the possible occurrence of bacteriocin producing *Lactobacillus* strains in the sample, the combination of sodium phosphate dibasic and monobasic is more suitable to be used as buffering salts to isolate bacteriocin producing LAB.

The three tested bacteriocin producing *Lactococcus lactis* strains showed larger diameter of inhibition zones with an increase in bottom media pH, which might suggest that those strains have higher bacteriocin production at initial pH 6.9. The reason for the inhibition zones formed by the bacteriocin non-producers at initial pH 5.5 was that those LAB might reduce their surrounding pH of bottom media to too low after 24 hours of incubation, which could inhibit the growth of the indicator strain *Listeria monocytogenes*. According to the test results, the appropriate initial pH of bottom media used in deferred antagonism assay should be 6.9. However, there are specific bacteriocin-producers that have different preferred pH. For example, the highest production of Lactocin S by *Lactobacillus sake* L45 was observed when it was grown at pH 5.0; the production of Lactocin S was only one-tenth of the highest level when the producer was grown at pH 6.0 (Mortvedt-Abildgaa et al., 1995). Parente et al. (1994) reported that the highest production of lactococcin 140 was observed at initial pH 5.5. To isolate those bacteriocin-producers from foods and prevent the isolation of bacteriocin non-producers, the bottom media at initial pH 6.0 is also required in deferred antagonism assay.

The addition of 1% Tween 80 significantly increased the diameter of inhibition zones, which means 1% Tween 80 can increase the bacteriocin production. Ravi et al. (2017), Jung et al. (1992), and Vignolo et al. (1995) also reported that Tween 80 could remarkably increase the production of bacteriocins by LAB. Vignolo et al. (1995) reported that Tween 80 was a critical factor in lactocin 705 production, which works as a surfactant on the cell membranes of its producer and can accelerate diffusion of lactocin 705. Jung et al. (1992) stated that Tween 80 may counteract the adsorption of the bacteriocin by proteins, thus making possible the inhibition of microorganisms. As for ethanol, although Mortvedt-Abildgaa et al. (1995) reported 1% ethanol can increase the bacteriocin yield, there was no significant difference in the diameter of inhibition zone caused by bacteriocin-producers tested in this study.

In conclusion, adding 1% Tween 80 into Elliker medium with the combination of sodium phosphate dibasic and monobasic at initial pH 6.9 or pH 6.0 can enhance the possibility of isolating bacteriocin producing LAB in the deferred antagonism assay. For future research, other factors, like the concentration of glucose, which can increase the production of bacteriocins by LAB should be considered and tested based on this study. There is a high demand for potent bacteriocins and strong bacteriocin-producers isolated from foods.

Chapter 4

Isolation, identification and characterization of bacteriocin producing lactic acid bacteria from foods

4.1 Introduction

Bacteriocins are ribosomally synthesized proteins produced by bacteria which are bactericidal or bacteriostatic. Bacteriocin production is a widespread phenomenon among lactic acid bacteria (LAB), having been observed among lactobacilli, lactococci, pediococci, and leuconostocs (Klaenhammer, 1988). In an environment with mixed bacterial populations, the production of bacteriocins is a competitive strategy which is advantageous for producing organisms to get more nutrients and space to grow. LAB are a critical microbial group for industrial applications They have been commonly utilized as starter cultures in fermented products sold on marketplaces, such as cheese, yogurt, and buttermilk. As a result, bacteriocins produced by LAB, used in fermented foods, are deemed novel, safe and natural food preservatives. Bacteriocins are sensitive to digestive protease and do not induce changes in the organoleptic properties of the foods (Cabo et al., 2001). Therefore, bacteriocin producing LAB can be isolated from fermented foods and applied to enhance food safety and quality.

Although bacteriocins can inhibit bacteria closely related to the bacteriocin-producers, studies have shown that when bacteriocinogenic strains are added to food, their antimicrobial effect is often less than expected (Mortvedt-Abildgaa et al., 1995). The reason is that the environment of those bacteriocinogenic strains in foods may reduce their production of bacteriocins. Hence, the growth condition of bacteria is important for bacteriocin production.

Maximal bacteriocin production can be achieved by using carefully selected culture medium with optimized pH and certain supplements, such as ethanol and Tween 80. Geis et al. (1983) reported that Elliker medium is better than M17 or milk for bacteriocin production. Plantaricin S, produced by *Lactobacillus plantarum* LPCO-10, which is involved in the fermentation of olives, reached a peak of antimicrobial activity in MRS broth (Mortvedt-Abildgaa, et al., 1995).

As for pH, Vignolo et al. (1995) reported that the production of lactocin 705 by *Lactobacillus casei* CRL 705 isolated from dry sausages reached the maximum at pH 6.5-7.5 in MRS broth. Meera and Devi (2017) showed that isolated bacteriocin producing LAB displayed maximum bacteriocin activity at pH 6 in MRS broth. Turgis et al. (2016) showed that the maximum nisin production of *Lactococcus lactis* MM19 was achieved at pH 9; the maximum pediocin production of *Pediococcus acidilactici* MM33 was achieved at pH 6. Mortvedt-Abildgaa et al. (1995) reported *Lactobacillus sake* L45 produced more bacteriocin at pH 5 than at pH 6.

Some supplements also have significant effects on the production of bacteriocin. Tween 80 is one of the surfactants which can provide essential oleate to metabolically active LAB (Jacques et al., 1980). The addition of Tween 80 (0.5%-2%) to growth medium can increase the production of lactocin 705, with the maximum bacteriocin activity at 1% Tween 80 (Vignolo et al., 1995). Ravi et al. (2017) also reported that the 1% Tween 80 can increase the production of bacteriocin from the LAB isolated from mango. Besides favorable components, specific bacteriocin production can be enhanced under unfavorable conditions such as low level of ethanol. De Vuyst et al. (1996) reported 1% ethanol can increase the production of

amylovorin L471, a bacteriocin produced by *Lactobacillus amylovorus*, by two-fold. Mortvedt-Abildgaa et al. (1995) showed that the addition of 1% ethanol to growth medium can cause two- to four-fold increase in the production of lactocin S.

In this study, a variety of fermented foods were used to isolate bacteriocin producing LAB via the improved deferred antagonism assay. After the isolation, the species 16S rRNA and the bacteriocin encoding genes carried by the bacteriocin producing LAB were identified. The main objective was to identify bacteriocin producing LAB that have inhibitory activity against foodborne pathogens. In addition, the influence of growth conditions of isolated LAB on their production of bacteriocins was determined, which included the culture medium, initial pH, and medium supplements.

4.2 Materials and Methods

4.2.1 Sample collection

Fermented kimchee, sauerkraut, yoghurt, and kefir were collected from local food stores. All food samples were stored at 7°C prior to be subjected to microbiological experiments in the laboratory.

4.2.2 Isolation of bacteriocin producing bacteria from foods

Ten grams of each sample were mixed with 90 ml 0.1% peptone water in sterile stomach bags before being homogenized at 200 rpm for 1.5 minutes. After that, the homogenates were serially 10-fold diluted with 9 ml peptone water. 100 µl of sample diluents were spread plated on MRS agar to identify appropriate dilutions at 250-2,500 CFU/ml. The bacteriocin

producing capability of bacteria in the sample was checked by the improved deferred antagonism assay described in Chapter 3. Appropriate sample dilutions were spread onto buffered Elliker medium (1% Na_2HPO_4 and 0.35% NaH_2PO_4) with 1% Tween 80. The pH of the medium was adjusted to 6.9 and 6.0 respectively by using 3 M hydrochloric acid. Plated samples were immediately covered with the same buffered Elliker medium and incubated at 32 ℃ for 24 to 48 hours until the colony showed up. Then, the indicator strain, *listeria monocytogenes* (10^7 CFU), was mixed with molten BHI agar (0.75% agar) and covered on the second agar layer. During incubation at 32℃ for 24 hours, indicator strain grew to compete with the bacteria from fermented food samples. Therefore, bacteriocin producing bacteria would show inhibition zones surround them. To confirm the production of bacteriocin, the colonies which showed the inhibition zones were picked by using sterile needles. Then, those isolates were streaked on MRS agar to get the purified single colony. Each isolate was grown in MRS broth at 32℃ for 24 hours. The pure cultures were centrifuged at 4,000 rpm for 10 min, and their supernatants were neutralized to pH 5.8 by using 1 M sodium hydroxide. The neutralized supernatants were filter-sterilized. 100 µl of 10^8 CFU/ml *Listeria monocytogenes* were added into each cell-free neutralized supernatant. The supernatants which kept clear after incubation at 32℃ for 24 hours represented the containment of bacteriocins.

4.2.3 Extraction of DNA from the isolates

Five hundred microliters of the pure cultures were pipetted into sterile 1.7-milliliter tubes. The tubes were centrifuged at 14,000 rpm for 2 min, and resulting pellets were used for DNA

extraction. DNA extraction was performed using the DNeasy Tissue kit (Qiagen, Maryland, USA) following the protocol for Gram-positive bacteria.

4.2.4 Identification of the isolates by 16S rDNA amplification and sequencing

To identify the isolates, their DNA extracts were amplified with universal primers 16S rRNA-F (5'-GGA GAG TTT GAT CCT GGC TCA G-3') and 16S rRNA-R (5'-TAT TAC CGC GGC TGC TGG CAC-3') targeting the eubacterial 16s rRNA gene (Vilegen et al., 2006). A total of 25 µl reaction mixture contained 12 µl Master mix (Promega, WI), 10 µl nuclease-free water, 0.5 µl each of forward and reverse primers, and 2 µl of sample DNA. The PCR reaction included an initial denaturation at 95℃ for 5 min, followed by 30 cycles of 94℃ for 30 s (denaturation), 62℃ for 30 s (annealing), 72℃ for 45 s (extension), and a final extension step at 72℃ for 10 min. The amplified samples were held at 10℃. The amplification products of PCR reaction were checked by gel electrophoresis. The PCR products were run in a 1.5% agarose gel in 1X TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) at 80V for 30 min. The gels were then stained with SYBR Green for 30 min and photographed by using the FOTO/Analyst investigator system. All PCR reactions in this study included a negative control containing the same PCR mixture without template DNA, which was run in parallel to determine potential occurrence of false positive results.

The amplified DNA was cleaned of excess primers and dNTPs with ExoSAP-IT before sequencing. 5 µl PCR products were mixed with 2 µl of ExoSAP-IT reagent completely. The mixture was held at 37℃ for 15 min, followed by inactivation of ExoSAP-IT via heating at 80℃ for 15 min. 5 µl of purified amplified DNA from each isolate were mixed with 0.5 µl of

the forward or reverse primer and 6.5 µl nuclease-free water. The mixture was submitted to the Biotechnology Core Facility of the University of Hawaii at Manoa for sequencing. The sequences were identified using the National Center for Biotechnology Information (NCBI) Nucleotide BLAST Program.

4.2.5 Differentiation of the isolates via RAPD-PCR

Although PCR reaction and sequencing can identify the isolates to the species level, the genetic distinction of individual strain still needed to be determined by using random amplification of polymorphic DNA (RAPD)-PCR. The M13 primer (5'-GAG GGT GGC GGT TCT-3') was used for amplification of template DNA in RAPD-PCR according to the protocol previously described by Pulido et al. (2005). A volume of 25 µl of reaction mixture contained 12.5 µl Master mix, 10 µl nuclease-free water, 0.5 µl of 50 mM M13 primer, and 2 µl DNA extracted from the isolated LAB. The amplification program included an initial denaturation at 95℃ for 5 min, followed by 35 cycles of 94℃ for 1 min (denaturation), 40℃ for 20 s (annealing), ramp to 72℃ at 0.6℃/s, and hold at 72℃ for 2 min (extension). A final extension step was carried out at 72℃ for 10 min. 5 µl of RAPD-PCR products were analyzed by gel electrophoresis. The amplified products were run in a 1.5% agarose gel in 0.5X TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) at 55V for 10 hours. The 100 bp Plus DNA ladder was used as a molecular weight marker for reference. The gels were then stained with SYBR Green for 30 min and photographed by using the FOTO/Analyst investigator system. RAPD-PCR banding patterns of all LAB isolates were obtained and analyzed. Isolates with the same banding pattern were considered to be the same strain.

4.2.6 Detection of bacteriocin genes of the isolates

A bacteriocin-specific PCR array using eight pairs of *Lactococcus lactis* bacteriocin gene primers, designed by Macwana et al. (2012), and three pairs of *Lactobacillus plantarum* bacteriocin gene primers, described by Barbosa et al. (2016), was employed to determine bacteriocin genes of the isolates (Table 2). A total 25 µl of reaction mixture contained 12.5 µl Master mix, 10 µl nuclease-free water, 0.25 µl of forward and reverse primers from an individual pair, and 2 µl DNA extracted from the isolated LAB. The PCR reactions with positive results were run one more time to confirm. The amplification program included an initial denaturation at 94℃ for 5 min, followed by 30 cycles of 94℃ for 30 s (denaturation), annealing at the temperature shown in table 2 for 30 s, and hold at 72℃ for 30s (extension). A final extension step was carried out at 72℃ for 5 min. Amplicons showing positive results through the agarose gel electrophoresis analysis were sent out for sequencing in both directions. Amplicon sequences were analyzed to determine their similarity to bacterocin-encoding gene sequences available in the NCBI GenBank.

4.2.7 Determination of the inhibitory spectrum of bacteriocins produced by the isolates

100 µl of 10^8 CFU/ml pathogenic bacteria, including *Salmonella* Typhimurium, *Staphylococcus aureus* and *E. coli* O157:H7, were individually evaluated for their sensitivity to selected LAB isolates via the improved deferred antagonism assay.

Table 2. List of primers used for the bacteriocin-specific PCR targeting LAB

Organism	Primers	Sequences	Annealing Temp (ºC)	Size (bp)
Lactococcus lactis subsp. *lactis*	IclA-F	aaaccaagtctctcgtattggc	60	200
	IclA-R	ggcacgttgtgtatccttacct		
Lactococcus lactis	LtnA-F	ccagttacatggttggaagaag	56.4	150
	LtnA-R	tttacaccaagccatacattca		
Lactococcus lactis subsp. *lactis*	IcnB-F	agttaatggaggaagcttgcag	57.7	156
	IcnB-R	tagtggaatgtttttccccatc		
Lactococcus lactis	LtnB-F	caattgggaaaataccttgaaga	56.4	152
	LtnB-R	caagcacgtgtacattttgttgt		
Lactococcus lactis subsp. *lactis*	LacA-F	agtgctattcaaaattctggcg	57.7	217
	LacA-R	taatccaacctccggaataaga		
Lactococcus lactis subsp. *cremoris*	IcpJ-F	tggaccttattttaggtgcaaaa	57.7	100
	IcpJ-R	gagcagcaagtaaatacaaagttcc		
Lactococcus lactis	NisZ-F	atgagtacaaaagattttaacttgg	56.4	174
	NisZ-R	ttatttgcttacgtgaatactaca		
Lactococcus lactis KF147	Bac147-F	accaatgtttggtttgaactgta	54	314
	Bac147-R	aaaattacttgtttcaacacttatcct		
Lactobacillus plantarum LPCO 10	PlnS-F	gccttaccagcgtaatgccc	58	450
	PlnS-R	ctggtcatgcaatggttagttt		
Lactobacillus plantarum	PlnNC8-F	ggtctgcgtataagcatcgc	51	207
	PlnNC8-R	aaattgaacatatgggtgctttaaaatcc		
Lactobacillus plantarum	PlnW-F	tcacacgaaatattcca	50	165
	PlnW-R	ggcaagcgtaagaaataaatgag		

4.2.8 Optimal conditions for bacteriocin production by representative isolates

Representative strains from distinct groups of the isolates determined by RAPD PCR banding patterns were grown at 32℃ for 24 hours in three types of medium, including MRS broth, M17 broth, and Elliker broth. The fresh cultures were centrifuged at 4,000 rpm for 10 min. Then the supernatants were neutralized to pH 5.8 with 1 M sodium hydroxide and filter-sterilized. The antimicrobial activity of bacteriocins produced by representative isolates was quantified using a micro-titer plate assay system described by Mørtvedt et al (1991). 100 µl of bacteriocin extractions were serially diluted with 100 µl of broth, the same type as the one used in culturing the strain, in 0.65 ml centrifuge tubes. 20 µl of *Listeria monocytogenes* culture mixed with 80 µl of the same broth were also added to the centrifuge tubes. The tubes were incubated at 32℃ for 24 hours, and the mixtures were transferred into 12X8 micro-titer plates. Growth inhibition of *Listeria monocytogenes* was measured spectrophotometrically at 600 nm. One bacteriocin unit (BU) was defined as the amount of bacteriocin which inhibited the growth of *Listeria monocytogenes* by 50% (50% of the turbidity of the control culture without bacteriocin) under standard assay conditions. The antimicrobial activities of bacteriocins produced by each representative isolate grown in three types of broth were determined and compared. After the most appropriate medium was identified, the influence of initial medium pH on the bacteriocin production was determined. Finally, 1% Tween and 1% ethanol were added individually and in combination to the medium with optimal pH. The bacteriocin production of each representative isolate in these supplemented media was measured and compared.

4.3 Results

4.3.1 Isolation of bacteriocin-producers from foods

The tested food samples yielded colonies forming inhibition zones in the deferred antagonism assay (Figure 9). A total of 10 colonies were picked and cultivated in MRS broth. Their cell-free neutralized supernatants remained clear after being inoculated with *Listeria monocytogenes* cells and cultured for 24 h.

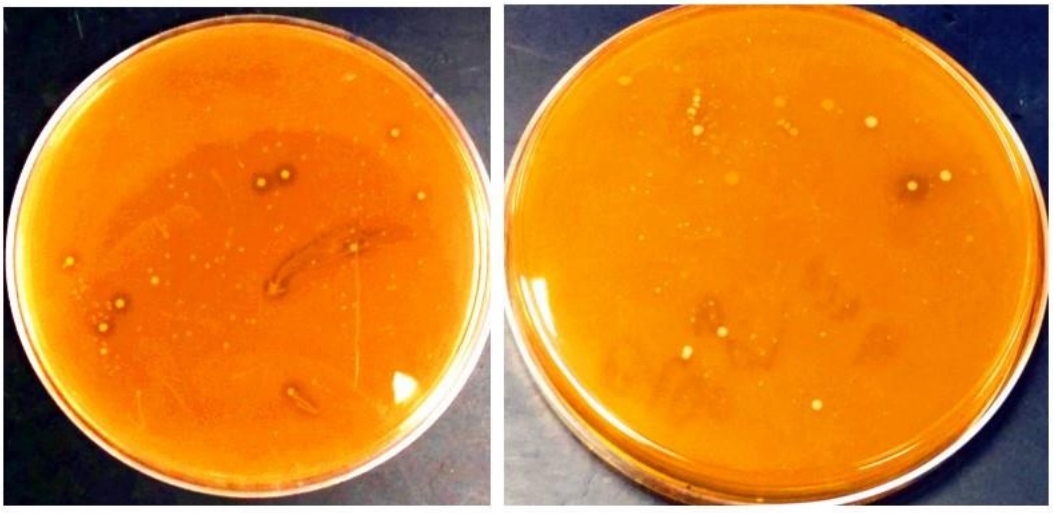


Figure 9. Inhibition zones caused by lactic acid bacteria isolated from the fermented foods

4.3.2 Identification of isolated LAB

PCR amplification of DNA from the 10 isolates with the 16S RNA gene primers generated amplicons of 500 bp (data not shown), which was the same as expected size. Sequencing analysis disclosded eight isolates belonged to *Lactococcus lactis* and the other two belonged to *Lactobacillus plantarum* (Table 3).

Table 3. Sequencing information for bacteriocin producing LAB isolates

Isolate ID	Closest Relative	% Identify	GenBank No.
#1, #2, #3, #4, #5	*Lactococcus lactis* subsp. *lactis*	99%	LC311732.1
#6	*Lactococcus lactis*	98%	MF185375.1
#7, #8	*Lactococcus lactis* subsp. *lactis*	98%	MF369874.1
#9, #10	*Lactobacillus plantarum*	99%	CP024413.1

4.3.3 Differentiation of the bacteriocin producing LAB isolates

The gel of RAPD PCR is exhibited in Figure 10. The amplicons from 10 isolates were run at the same time. Isolates 1 to 8 were *Lactococcus lactis*, and the last two were *Lactobacillus plantarum*. The profiles of all LAB isolates were compared to one another in hope of differentiating isolates with unique binding patterns and grouping those with identical profiles as the same strain. The group 1 included isolates 1, 2, 4, and 5 which showed the same four amplicons of 1400 to 2200 bp. These four isolates are considered the same strain. The group 2 was isolates 3 which displayed three amplicons of 1400 to 2000 bp. The group 3 was isolates 6 which showed two amplicons of 1400 to 2000 bp. The group 4 included isolates 7 and 8 which had four amplicons of 1400 to 2200 bp. The group 5 included isolates 9 and 10 with 7 DNA amplicons of 1000 to 3200 bp. Therefore, a total of four types of *Lactococcus lactis* and one type of *Lactobacillus plantarum* were isolated from the fermented foods. The isolate #1, #3, #6, #7 and #9 were chosen as representative strains and used in the following tests.

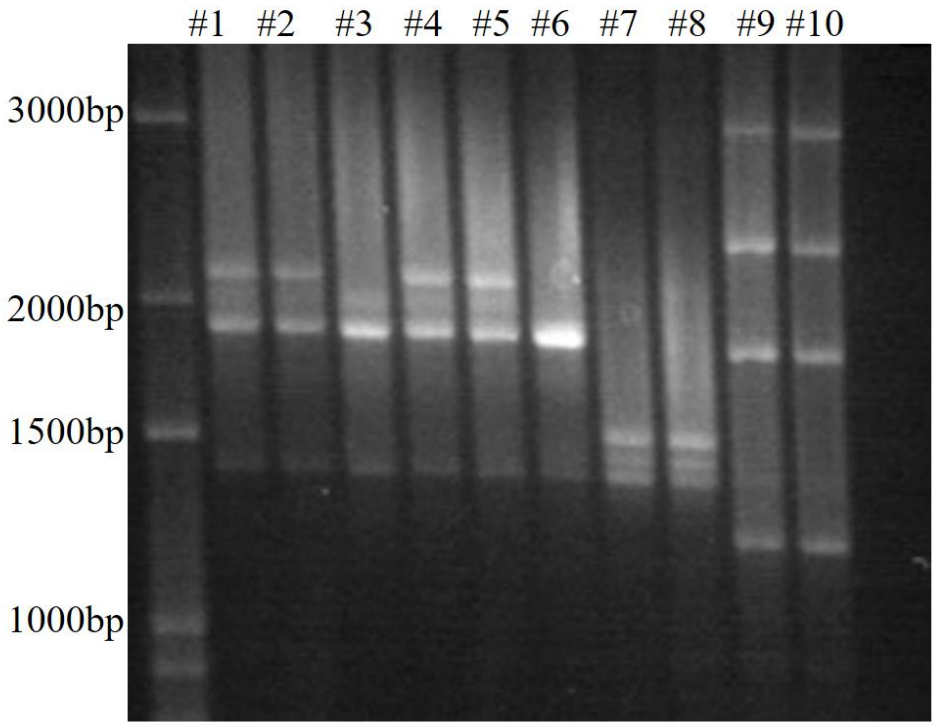
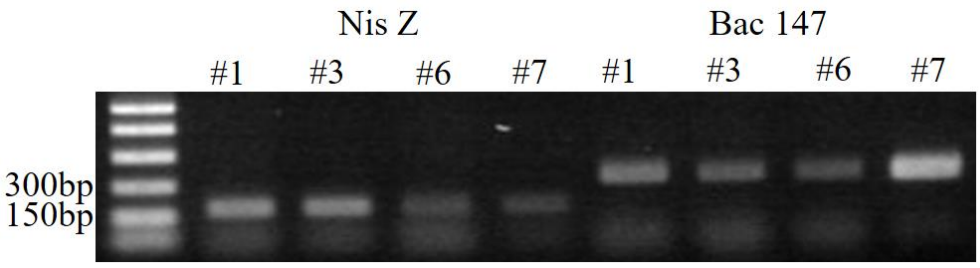


Figure 10. RAPD-PCR gel profiles for bacteriocin producing LAB isolates from the fermented foods

4.3.4 Identification of bacteriocin genes of representative isolates

For all four types of *Lactococcus lactis* isolates, two sets of primers, nisin Z and Bac 147, showed positive results in PCR amplification. They yielded about 200 bp and 300 bp products, respectively (Figure 11A). The sequencing results confirmed that they covered the nisin Z and lactococcin 972 genes (Table 4). As to the *Lactobacillus plantarum*, the plantaricin S primers could bind to its DNA during annealing. PCR amplification generated a 450 bp amplicon (Figure 11.B). When the DNA sequence generated by the primer set PlnS was compared with that of *Lactobacillus plantarum* LPCO10, which was the strain used to design the primers, there were obvious differences between the intra-primer regions. However, high homology of the amplicon to the genome of *Lactobacillus plantarum* strain PC520 was observed (Table 4).

A



B

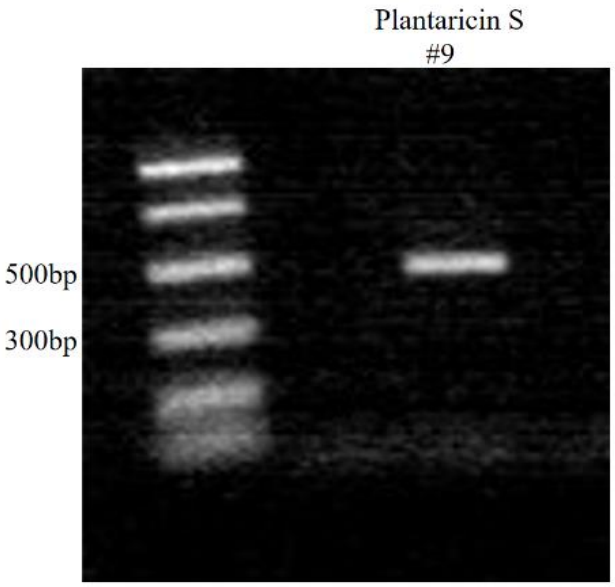


Figure 11. Agarose gel electrophoresis of PCR amplicons generated from DNA of *Lactococcus lactis* isolates with primer sets Nis Z and Bac 147 (A), and DNA of *Lactobacillus plantarum* isolate with primer set Pln S (B)

Table 4. Identification of bacteriocin genes of representative LAB isolates amplified by primer sets Nis Z, Bac 147 and Pln S

Isolate ID	Primers	Closest Relative	Identity	GenBank Accession No.
#1, #3, #6, #7	Nis Z	*Lactococcus lactis* strain 806 nisin Z (nisZ) gene	99%	KY496773.1
#1, #3, #6, #7	Bac 147	lactococcin 972 family bacteriocin [*Lactococcus lactis*]	99%	WP_029344490.1
#9	Pln S	*Lactobacillus plantarum* strain PC520 genome	99%	CP023772.1

4.3.5 The inhibition spectrum of representative isolates

Lactococcus lactis isolates #1, #3, and #7 also showed antimicrobial activity against *Staphylococcus aureus*. However, isolated #6 *Lactococcus lactis* and #9 *Lactobacillus plantarum* could not inhibit the growth of *Staphylococcus aureus*. None of the isolates showed inhibition against gram-negative pathogens *Salmonella* Typhimurium and *E. coli* O157:H7.

4.3.6 The effect of growth conditions on bacteriocin production of representative isolates

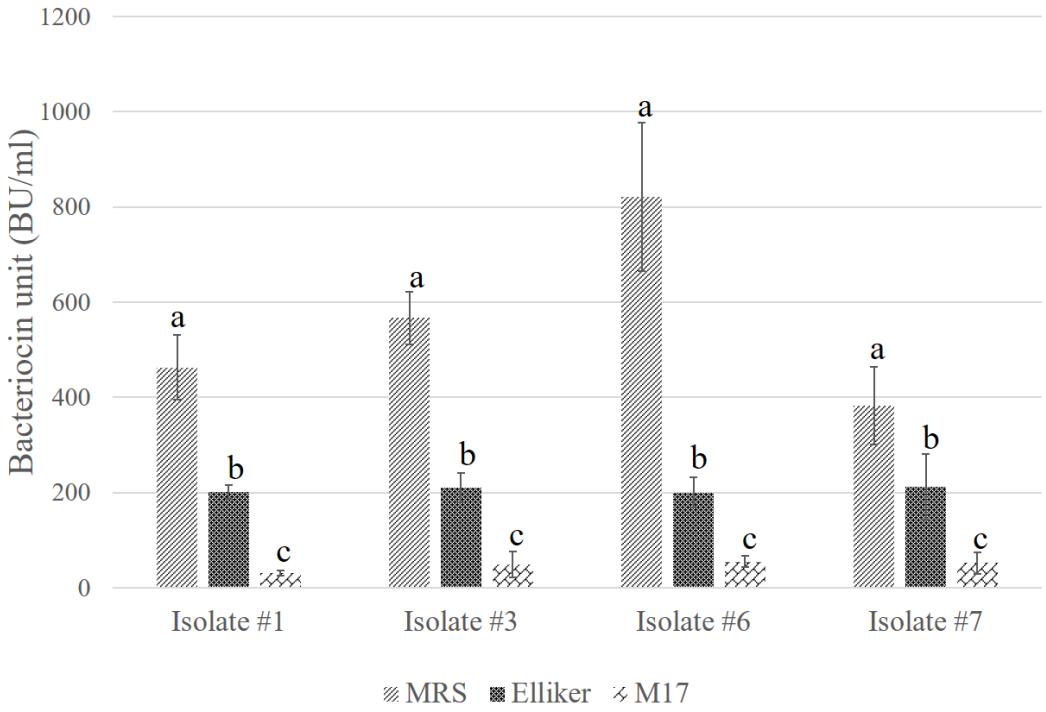
The influence of culture medium on the bacteriocin production was demonstrated in Figure 8. For *Lactococcus lactis* isolates, the highest bacteriocin activity was observed when they were grown in MRS broth (Figure 12A). The bacteriocin production of isolates #1, #3 and #6 was doubled in MRS broth compated to Elliker broth. When those *Lactococcus lactis* isolates were grown in M17 broth, their bacteriocin production was significantly lower. As for the isolated *Lactobacillus plantarum* #9, its antimicrobial activity to *Listeria monocytogenes* was much lower than that of the *Lactococcus lactis* isolates, which only showed around 13 BU/ml (Figure 12B). There was no significant difference in its antimicrobial activity when the isolate was grown in those different culture media.

The initial pH of MRS broth showed remarkable effects on the production of bacteriocins (Figure 13A and Figure 13B). All tested representative isolates had higher bacteriocin production with increasing pH. Highest bacteriocin production of isolate #6 was observed at pH 6.9 (1059 BU/ml) which was 5-fold higher than its bacteriocin production at pH 5.5. The bacteriocin production of isolated *Lactobacillus plantarum* #9 had no significant change until

the medium pH reached 6.9, which was more than double of the production at pH 6.0.

As shown in Figure 14, supplements added to MRS broth at pH 6.9 could affect the production of bacteriocins. The addition of 1% Tween 80 promoted bacteriocin production by all representative isolates. The highest bacteriocin production (1316 BU/ml) was achieved by isolate #6 with 1% Tween 80. The bacteriocin production of isolated *Lactococcus lactis* #1, #3, #6 and #7 did not show significant change when 1% ethanol was added to the culture medium (Figure 14A). However, 1% ethanol noticeably increased the bacteriocin yield of isolated *Lactobacillus plantarum* #9 by 7-fold, which was higher than the effect of 1% Tween 80 (Figure 14B). Besides, adding the combination of 1% Tween 80 and 1% ethanol had no increased effect than only adding 1% Tween 80 on the bacteriocin production of *Lactococcus lactis* isolates or 1% ethanol on the bacteriocin production of *Lactobacillus plantarum*.

A



B

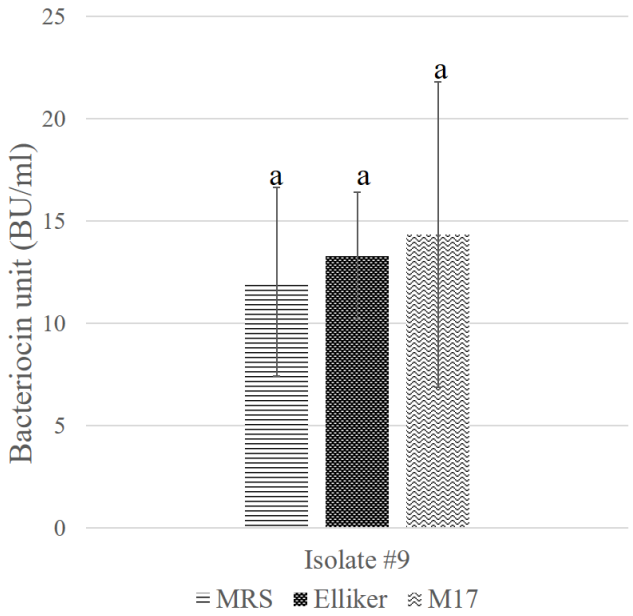
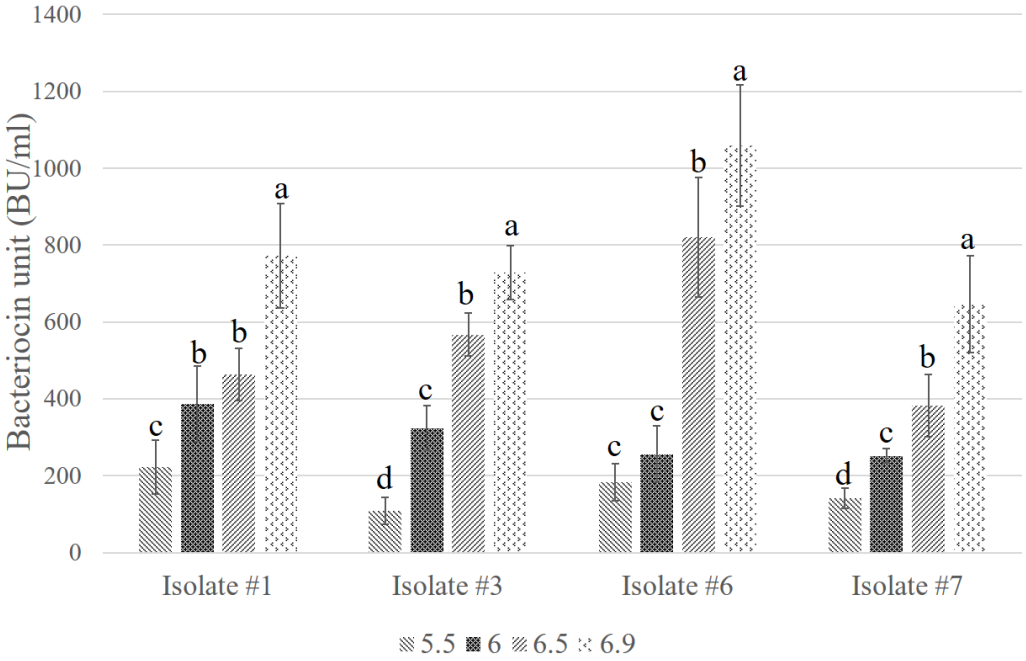


Figure 12. Influences of medium on bacteriocin production (BU/ml) by Lactococcus lactis isolates (A) and Lactobacillus platarum isolates (B)

A



B

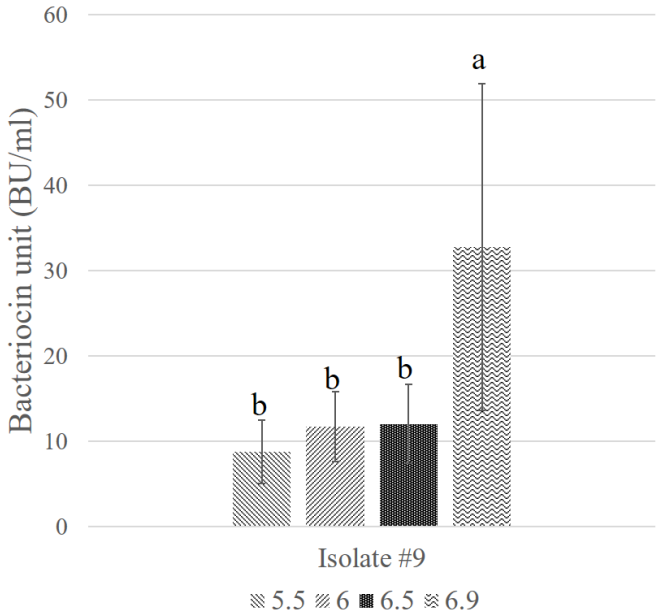
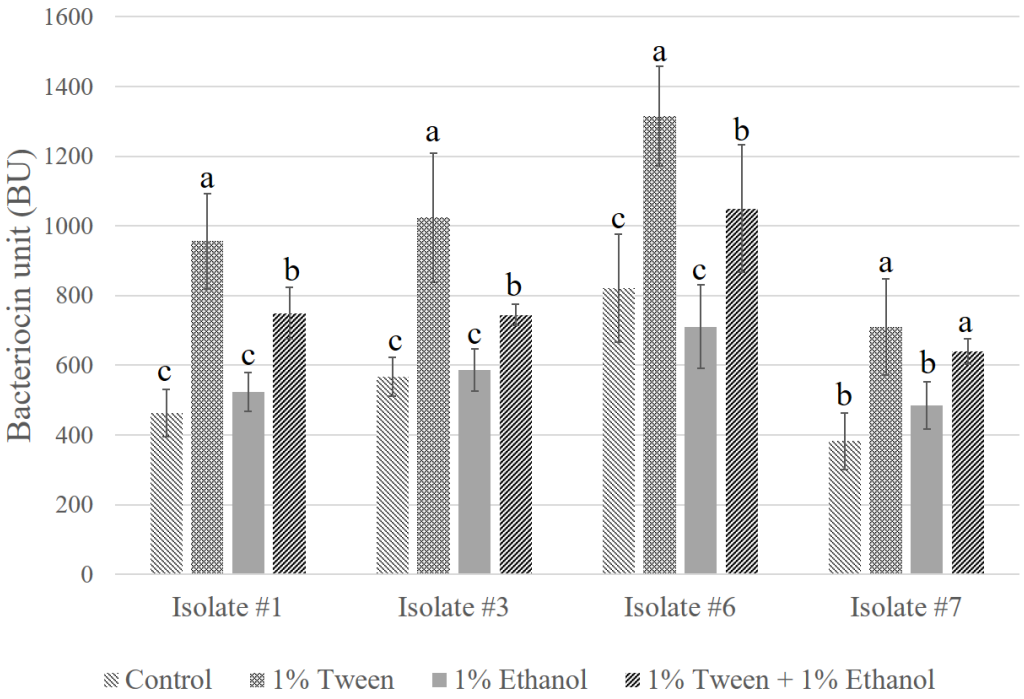


Figure 13. Influences of initial medium pH on bacteriocin production (BU/ml) by Lactococcus lactis isolates (A) and Lactobacillus platarum isolates (B)

A



B

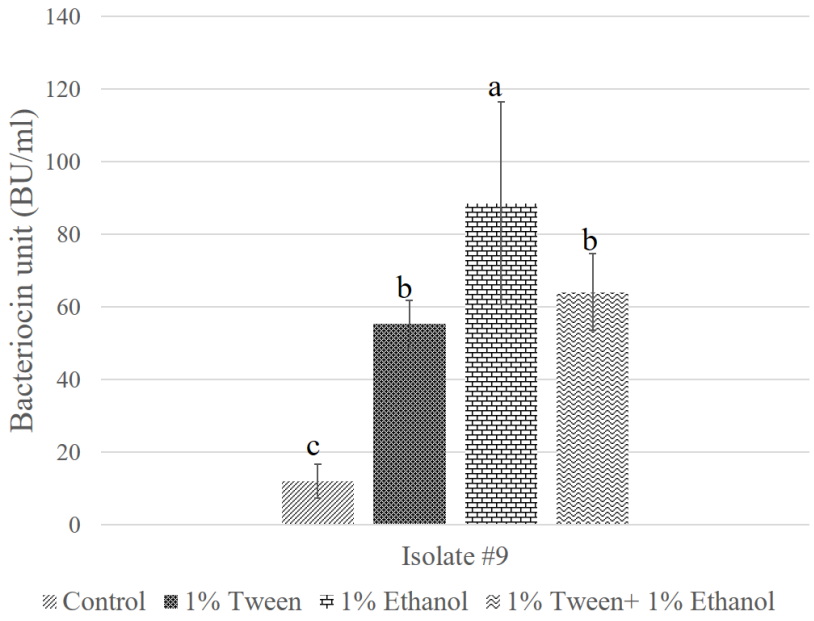


Figure 14. Influences of supplements on bacteriocin production (BU/ml) by Lactococcus lactis isolates (A) and Lactobacillus platarum isolates (B)

4.4 Discussions

A total of 10 bacteriocin-producers were isolated from the food samples, demonstrating the effectiveness of the improved deferred antagonism assay. As Figure 6 shows, the bacteriocin-producers did not dominate the microbiota of fermented foods. The PCR amplification and DNA sequencing suggest that those bacteriocin-producers belonged to *Lactococcus lactis* and *Lactobacillus plantarum*, which were common starter cultures used in fermented foods. Four different types of *Lactococcus lactis* and one type of *Lactobacillus plantarum* were identified among those 10 isolates by using RAPD PCR. The reason why the amplicons of RAPD PCR had longer size than those generated in general PCR was that its extension time was over 3-fold longer than that in general PCR. The amplification of the representative isolates with bacteriocin gene primers showed that all *Lactococcus lactis* isolates carried the nisin Z and lactococcin 972 genes, and isolated *Lactobacillus plantarum* carried the plantaricin S gene. The lactococcin 972 had a narrow inhibition spectrum, effective only against sensitive lactococci (Bacillus 1998). Therefore, the antimicrobial effect of isolated *Lactococcus lactis* on *Listeria monocytogenes* was due to the secretion of nisin Z. The isolates #1, #3, and #7 could also inhibit the growth of *Staphylococcus aureus,* which was probably due to the expression of nisin Z gene (Pinto et al., 2011). However, the nisin Z gene carrier, isolate #6, could not inhibit *Staphylococcus aureus,* suggesting that bacteria possessing the same bacteriocin gene may not necessarily express the same antimicrobial effect.

According to the results, culture medium had significant effects on the bacteriocin production of representative isolates. Isolated *Lactococcus lactis* generated more bacteriocins in MRS broth than Elliker broth or M17 broth. The possible reason was that MRS broth

contains 0.1% Tween 80 which acts as a surfactant and can increase the permeability of cell membrane to promote bacteriocin production (Vignolo et al., 1995). Jung et al. (1992) also reported an enhanced effect on nisin activity in milk by emulsifier Tween 80, which counteracted the absorption of the bacteriocin by proteins, thus increased the effect of nisin. In addition, the concentration of nitrogen sources, peptone and yeast extract, are higher in MRS broth than in M17 broth. Those nitrogen sources can promote the production of nisin (Cheigh et al., 2002). As for higher bacteriocin production of *L. lactis* in Elliker broth than in M17 broth, the abundant carbohydrate source in Elliker broth might make significant contributions. Although the concentration of glucose in Elliker broth is the same as M17 broth, there are extra 0.5% lactose and 0.5% sucrose in Ellker broth. Cheigh et al. (2002) confirmed that *Lactococcus lactis* produce more bacteriocin in M17 broth supplemented with lactose than the original M17 broth. Kim et al. (2006) also reported the addition of lactose to growth medium can increase the production of bacteriocins.

For *Lactobacillus plantarum*, Todorov and Dicks (2005) confirmed that their bacteriocin production was higher in MRS broth than in M17 broth. That was not observed with the isolate in this study. One reason could be the bacteriocin produced by the isolated *Lactobacillus plantarum* was not inhibitive enough to the indicator organism *Listeria monocytogenes*, which might interfere with determining the influence of culture medium on the production of plantaricin S.

In this study, increasing initial pH of culture medium could enhance the bacteriocin production of all representative isolates. When the initial pH reached 6.9, the highest bacteriocin production of isolated *Lactococcus lactis* and *Lactobacillus plantarum* was

observed. For *Lactococcus lactis*, this result was different from the finding of Matsusaki et al. (1996). Matsusaki et al. reported that the optimal nisin production pH was around 5 to 5.5. However, Turgis et al. (2016) reported that the optimal nisin production pH was in the range from 7 to 9. The production of plantaricin S had the same problem. Todorov et al. (1999) announced that the highest production of plantaricin S was achieved at pH 6. However, in 2005, Todorov reported that there was no difference observed for plantaricin S production at pH 5.5, 6 and 6.5. Therefore, the same species of LAB isolated from different food samples may have different pH requirements for bacteriocin production.

The results showed that adding 1% Tween 80 into culture medium can increase the production of nisin and plantaricin S. This is similar to the findings of Vignolo et al. (1995), Jung et al. (1992), and Malheiros et al. (2015). In comparison, the addition of 1% ethanol only increased the production of plantaricin S. The reason was that low concentration of ethanol could cause slightly unfavorable growth conditions which might increase the bacteriocin production (De Vuyst et al., 1996) and/or prevent the bacteriocin aggregation (Mortvedt-Abildgaa et al., 1995). However, there was no significant influence of 1% ethanol on the nisin production. This might be explained by the fact that *Lactococcus lactis* has lower ethanol tolerance. The maximum ethanol tolerance of *Lactococcus lactis* is about 7% (Hviid et al., 2017). But *Lactobacillus plantarum* can still proliferate with 13% ethanol (G-Alegría et al., 2004). The high ethanol tolerance of *Lactobacillus plantarum* ensures that 1% ethanol just generates slightly stressful conditions and even increases the permeability of its cell membrane (Hviid et al., 2017). However, 1% ethanol may inhibit the growth of *Lactococcus lactis*, and higher ethanol concentration can decrease its production of nisin (Müller-Auffermann et al.,

2015).

In conclusion, the improved deferred antagonism assay effectively isolated five types of bacateriocin-producing LAB from the fermented foods. The isolated *Lactococcus lactis* strains carry the nisin Z and lactococcin 972 gene, and can inhibit *Listeria monocytogenes* and *Staphylococcus aureus* by producing nisin Z. The isolated *Lactobacillus plantarum* can also inhibit *Listeria monocytogenes* by producing plantaricin S. Bacteriocin production by the LAB isolates was significantly affected by the type and acidity of broth and supplements. Increasing the medium pH can enhance the bacteriocin production of all isolates. The yield of bacterions produced by *Lactococcus Lactis* and *Lactobacillus plantarum* significantly increased with the addition of 1% Tween 80. The supplement 1% ethanol only increased the bacteriocin production by *Lactobacillus plantarum*.

Lactococcus Lactis and *Lactobacillus plantarum* have been extensively applied in fermented food as starter cultures. Those bacteriocin-producers isolated in this study should be considered safe strains to use in fermented foods. Moreover, those isolates hold high potential to be developed as probiotics with valuable application in human health and animal production. In food manufacture, the isolated LAB and bacteriocins they produce can also be utilized as part of the hurdle technology to improve the quality and safety of food.

Chapter 5

Conclusions

There have been increased consumer demands for natural food preservatives. The discovery of bacteriocins meets the need since they may not only inhibit undesirable bacteria in food but also enhance human gut health. Research is progressing to find new bacteriocin-producers and explore their applications. Although there have been several approaches to isolating bacteriocin producing LAB, those methods are inefficient and may be interfered by certain bacteriocin non-producers which can produce other antimicrobial compounds.

In this study, the bacteriocin-producer isolation method, deferred antagonism assay, was improved by optimizing the bottom media composition. The replacement of MRS agar with Elliker agar as the bottom media in deferred antagonism assay eliminated the possibility of isolating bacteriocin non-producing LAB. The inhibition zones formed by bacteriocin-producers enlarged by adding 1% Tween 80 to the bottom media and increasing media initial pH. This improved method can decrease the interference of lactic acid produced by bacteriocin non-producing LAB and increase the effect of bacteriocins in the inhibition test. Hence, it would enhance the chance of isolating true bacteriocin-producers from foods.

By using the improved deferred antagonism assay, four types of bacteriocin producing *Lactococcus lactis* and one type of bacteriocin producing *Lactobacillus plantarum* were isolated from fermented foods including yoghurt, kefir, sauerkraut, and kimchee. All *Lactococcus lactis* isolates carry the nisin Z and lactococcin 972 genes, and the *Lactobacillus plantarum* isolate may carry a novel plantaricin S gene. Three types of isolated *Lactococcus lactis* can inhibit the growth of indicator pathogen, *Listeria monocytogenes,* and also show

antimicrobial activity against *Staphylococcus aureus*.

The bacteriocin production of the LAB isolates was affected by the type and acidity of broth medium and supplements. The maximum bacteriocin production of isolated *Lactococcus lactis* strains was achieved when they were cultivated in MRS broth with 1% Tween 80 at pH 6.9. As for *Lactobacillus plantarum*, culture medium did not show a significant effect on its bacteriocin production. The production of plantaricin S was more effective with increasing pH and supplements of 1% Tween 80 or 1% ethanol. 1% ethanol showed more significant effect on the plantaricin S production than 1% Tween 80.

The isolated LAB are commonly found in fermented foods. *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* are important starter cultures in dairy fermentation (Hayes et al., 2006). *Lactobacillus plantarum* is one kind of beneficial bacteria found in Kimchee, sauerkraut, cultured vegetables and even human saliva. Further research is required on these isolates before they can be directly used in foods. Although these isolates are naturally occurring bacteria and have been utilized in food fermentation without known negative effects, it would be wise to evaluate them for potential effect on humans. Upon confirmation that these isolates and their bacteriocins are safe, they could be utilized in foods to extend shelf life and improve quality. Moreover, the bacteriocin producing LAB isolates also hold a great potential to be applied as probiotics to improve gut health by secreting antimicrobial metabolites to fight off pathogens in the intestine. These isolated LAB should be tested for their capability to adhere to the human distal ileum and colon to elicit their health beneficial effects, and keep non-invasive to the harmless human commensal microbiota (Naidu et al., 1999).

Additional testing on the application of these LAB isolates is demanded as well. Their

antimicrobial influence on pathogens when they are added into foods or utilized to ferment foods has not been investigated. The effect of combinations of bacteriocins and other food preservation processes on pathogens, particularly gram-negative bacteria which are naturally resistant to the action of bacteriocins produced by gram-positive bacteria, is an interesting topic to investigate (Prudêncio et al., 2015).

This research has facilitated efficient isolation of bacteriocin producing LAB from fermented foods, identified five distinct bacteriocin-producers, and revealed important factors affecting their bacteriocin production. Research will continue on the application of the LAB isolates with a goal of confirming their safety and exploring their potential as probiotics. Their bacteriocins may one day be used as natural preservatives in food production.

Appendix A

Diameter of inhibition zones formed by the testing strains in Chapter 3

Table A.1 The effects of bottom media composition on the diameter of inhibition zones formed by bacteriocin-producing strains (Ki, L7-3 and B) and bacteriocin non-producing strains (R7-4, LGG and LBP)

The composition of bottom media	The diameters of inhibition zone (mm) of test strains					
	Ki	L7-3	B	R7-4	LGG	LBP
MRS + Na_2HPO_4 and NaH_2PO_4	3.25±0.27[a]	5.10±0.26[ab]	4.19±0.23[a]	1.75±0.35[a]	1.25±0.61[a]	1.81±0.60[a]
MRS + β-glycerophosphate	3.29±0.23[a]	5.16±0.19[a]	4.23±0.37[a]	1.63±0.70[a]	1.31±0.56[a]	1.88±0.78[a]
M17 + Na_2HPO_4 and NaH_2PO_4	3.13±0.95[a]	4.63±0.52[b]	3.75±0.27[b]	ND*	ND*	ND*
M17 + β-glycerophosphate	2.94±0.42[a]	3.75±0.65[c]	3.31±0.46[c]	ND*	ND*	ND*
Elliker + Na_2HPO_4 and NaH_2PO_4	3.38±0.69[a]	5.31±0.53[a]	4.17±0.63[a]	ND*	ND*	ND*
Elliker + β-glycerophosphate	3.56±0.32[a]	5.00±0.53[ab]	4.31±0.26[a]	ND*	ND*	ND*

*No inhibition zone detected

Table A.2 The effects of pH of bottom media on the diameter of inhibition zones formed by bacteriocin-producing strains (Ki, L7-3 and B) and bacteriocin non-producing strains (R7-4, LGG and LBP)

The initial pH of bottom media	The diameters of inhibition zone (mm) of test strains					
	Ki	L7-3	B	R7-4	LGG	LBP
pH 5.5	2.19±0.80^{b}	4.06±0.42^{b}	2.81±0.59^{b}	1.51±0.08	1.69±0.43	1.88±0.22
pH 6.0	2.63±0.44^{b}	4.00±0.46^{b}	3.06±0.50^{b}	ND*	ND*	ND*
pH 6.5	2.88±0.58^{ab}	4.81±0.65^{a}	3.69±0.46^{a}	ND*	ND*	ND*
pH 6.9	3.38±0.44^{a}	5.31±0.53^{a}	4.18±0.64^{a}	ND*	ND*	ND*

*No inhibition zone detected

Table A.3 The effects of tween 80 and/or ethanol on the diameter of inhibition zones formed by bacteriocin-producing strains (Ki, L7-3 and B) and bacteriocin non-producing strains (R7-4, LGG and LBP)

The supplement of bottom media	The diameters of inhibition zone (mm) of test strains					
	Ki	L7-3	B	R7-4	LGG	LBP
No Tween 80 or Ethanol (negative control)	3.38±0.70^{b}	5.31±0.53^{b}	4.18±0.64^{b}	ND*	ND*	ND*
1% Tween 80	4.39±0.66^{a}	6.09±0.32^{a}	5.48±0.43^{a}	ND*	ND*	ND*
1% Ethanol	3.31±0.53^{b}	5.25±0.46^{b}	4.13±0.59^{b}	ND*	ND*	ND*
1% Tween 80 and Ethanol	4.18±0.51^{a}	5.81±0.26^{a}	4.69±0.27^{a}	ND*	ND*	ND*

*No inhibition zone detected

Appendix B

Bacteriocin units of different isolates in Chapter 4

Table B.1 Influences of medium on bacteriocin production (BU/ml) by representative isolates

Type of medium	The bacteriocin units of representative isolates				
	#1	#3	#6	#7	#9
MRS	463.23±68.02[a]	567.12±55.51[a]	821.18±155.47[a]	382.04±81.68[a]	12.04±4.61[a]
Elliker	201.56±14.44[b]	210.47±31.16[b]	200.29±31.25[b]	212.39±69.32[b]	13.31±3.12[a]
M17	31.01±5.17[c]	49.31±26.51[c]	55.39±11.91[c]	51.88±22.72[c]	14.36±7.46[a]

Table B.2 Influences of initial medium pH on bacteriocin production (BU/ml) by representative isolates

Initial medium pH	The bacteriocin units of representative isolate				
	#1	#3	#6	#7	#9
6.9	771.65±13.60[a]	729.2±69.82[a]	1058.96±157.40[a]	645.45±125.88[a]	32.76±19.14[a]
6.5	463.23±68.02[b]	567.12±55.51[b]	821.18±155.47[b]	382.04±81.68[b]	12.04±4.61[b]
6	387.18±98.21[b]	324.08±58.64[c]	256.24±73.74[c]	251.64±19.28[c]	11.75±4.10[b]
5.5	222.75±69.57[c]	108.49±35.94[d]	183.11±48.92[c]	141.19±26.33[d]	8.76±3.70[b]

Table B.3 Influences of supplements on bacteriocin production (BU/ml) by representative isolates

Supplements	The bacteriocin units of representative isolate				
	#1	#3	#6	#7	#9
Control	463.23±68.02^{c}	567.12±55.51^{c}	821.18±155.47^{c}	382.04±81.68^{b}	12.04±4.61^{c}
1% Tween	955.84±136.17^{a}	1023.68±184.56^{a}	1315.8±142.94^{a}	709.99±138.32^{a}	55.32±6.52^{b}
1% Ethanol	524.20±55.73^{c}	587.69±60.52^{c}	711.19±120.57^{c}	484.51±67.15^{b}	88.59±27.79^{a}
1% Tween and 1% Ethanol	749.93±72.30^{b}	745.03±29.92^{b}	1050.1±182.46^{b}	639.55±35.82^{a}	64.13±10.63^{b}

References

Abriouel, H., Franz, C. M., Omar, N. B., & Gálvez, A. (2010). Diversity and applications of Bacillus bacteriocins. *FEMS microbiology reviews*, *35*(1), 201-232.

Adams, M. R., & Hall, C. J. (1988). Growth inhibition of food-borne pathogens by lactic and acetic acids and their mixtures. *International Journal of Food Science & Technology*, *23*(3), 287-292.

Ayad, E. H., Verheul, A., de Jong, C., Wouters, J. T., & Smit, G. (1999). Flavour forming abilities and amino acid requirements of Lactococcus lactis strains isolated from artisanal and non-dairy origin. *International Dairy Journal*, *9*(10), 725-735.

Bacillus, M. (1998). Detection and characterization of a bacteriocin produced by Lactococcus lactis subsp. cremoris R isolated from radish. *Letters in applied microbiology*, *26*, 297-304.

Bandell, M., Lhotte, M. E., Marty-Teysset, C., Veyrat, A., Prevost, H., Dartois, V., ... & Lolkema, J. S. (1998). Mechanism of the Citrate Transporters in Carbohydrate and Citrate Cometabolism in Lactococcus andLeuconostoc Species. *Applied and environmental microbiology*, *64*(5), 1594-1600.

Barbosa, M. S., Todorov, S. D., Ivanova, I. V., Belguesmia, Y., Choiset, Y., Rabesona, H., ... & Franco, B. D. G. M. (2016). Characterization of a two-peptide plantaricin produced by Lactobacillus plantarum MBSa4 isolated from Brazilian salami. *Food Control*, *60*, 103-112.

Beresford, T. P., Fitzsimons, N. A., Brennan, N. L., & Cogan, T. M. (2001). Recent advances in cheese microbiology. *International Dairy Journal*, *11*(4), 259-274.

Bergan, T., Solberg, R., & Solberg, O. (1984). 6 Fatty Acid and Carbohydrate Cell Composition in Pediococci and Aerococci, and Identification of Related Species. *Methods in microbiology*, *16*, 179-211.

Blaser, M. (2011). Antibiotic overuse: stop the killing of beneficial bacteria. *Nature*, *476*(7361), 393-394.

Brink, B. T., Minekus, M., Van der Vossen, J. M. B. M., Leer, R. J., & Veld, J. H. J. (1994). Antimicrobial activity of lactobacilli: preliminary characterization and optimization of production of acidocin B, a novel bacteriocin produced by Lactobacillus acidophilus M46. *Journal of Applied Microbiology*, *77*(2), 140-148.

Cabo, M. L., Murado, M. A., aP González, M., & Pastoriza, L. (2001). Effects of aeration and pH gradient on nisin production.: A mathematical model. *Enzyme and Microbial Technology*, *29*(4), 264-273.

Cai, Y., Kumai, S., Ogawa, M., Benno, Y., & Nakase, T. (1999). Characterization and

Identification ofPediococcus Species Isolated from Forage Crops and Their Application for Silage Preparation. *Applied and environmental microbiology*, *65*(7), 2901-2906.

Callewaert, R., Holo, H., Devreese, B., Van Beeumen, J., Nes, I., & De Vuyst, L. (1999). Characterization and production of amylovorin L471, a bacteriocin purified from Lactobacillus amylovorus DCE 471 by a novel three-step method. *Microbiology*, *145*(9), 2559-2568.

Castro MP, Palavecino NZ, Herman CO *et al.* (2011) Lactic acid bacteria isolated from artisanal dry sausages: Characterization of antibacterial compounds and study of the factors affecting bacteriocin production. Meat Sci 87:321–329

Cheigh, C. I., Choi, H. J., Park, H., Kim, S. B., Kook, M. C., Kim, T. S., ... & Pyun, Y. R. (2002). Influence of growth conditions on the production of a nisin-like bacteriocin by Lactococcus lactis subsp. lactis A164 isolated from kimchi. *Journal of Biotechnology*, *95*(3), 225-235.

Cogan, T. M., Barbosa, M., Beuvier, E., BIANCHI-SALVADORI, B. R. U. N. A., COCCONCELLI, P. S., FERNANDES, I., ... & MEDINA, M. (1997). Characterization of the lactic acid bacteria in artisanal dairy products. *Journal of Dairy Research*, *64*(3), 409-421.

Cotter, P. D., Hill, C., & Ross, R. P. (2005). Bacteriocins: developing innate immunity for food. *Nature Reviews Microbiology*, *3*(10), 777-788.

Cotter, P. D., Stanton, C., Ross, R. P., & Hill, C. (2012). The impact of antibiotics on the gut microbiota as revealed by high throughput DNA sequencing. *Discovery medicine*, *13*(70), 193-199.

De Carvalho, A. A. T., Vanetti, M. C. D., & Mantovani, H. C. (2008). Bovicin HC5 reduces thermal resistance of Alicyclobacillus acidoterrestris in acidic mango pulp. *Journal of applied microbiology*, *104*(6), 1685-1691.

De Vuyst, L., Callewaert, R., & Crabbe, K. (1996). Primary metabolite kinetics of bacteriocin biosynthesis by Lactobacillus amylovorus and evidence for stimulation of bacteriocin production under unfavourable growth conditions. *Microbiology*, *142*(4), 817-827.

Drider, D., Fimland, G., Héchard, Y., McMullen, L. M., & Prévost, H. (2006). The continuing story of class IIa bacteriocins. *Microbiology and molecular biology reviews*, *70*(2), 564-582.

Eijsink, V. G., Axelsson, L., Diep, D. B., Håvarstein, L. S., Holo, H., & Nes, I. F. (2002). Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Antonie Van Leeuwenhoek*, *81*(1-4), 639-654.

Ennahar, S., Cai, Y., & Fujita, Y. (2003). Phylogenetic diversity of lactic acid bacteria

associated with paddy rice silage as determined by 16S ribosomal DNA analysis. *Applied and Environmental Microbiology*, *69*(1), 444-451.

Ennahar, S., Sashihara, T., Sonomoto, K., & Ishizaki, A. (2000). Class IIa bacteriocins: biosynthesis, structure and activity. *FEMS microbiology reviews*, *24*(1), 85-106.

G-Alegría, E., López, I., Ruiz, J. I., Sáenz, J., Fernández, E., Zarazaga, M., ... & Ruiz-Larrea, F. (2004). High tolerance of wild Lactobacillus plantarum and Oenococcus oeni strains to lyophilisation and stress environmental conditions of acid pH and ethanol. *FEMS microbiology letters*, *230*(1), 53-61.

Garneau, S., Martin, N. I., & Vederas, J. C. (2002). Two-peptide bacteriocins produced by lactic acid bacteria. *Biochimie*, *84*(5), 577-592.

Geis, A., Singh, J., & Teuber, M. (1983). Potential of lactic streptococci to produce bacteriocin. *Applied and Environmental Microbiology*, *45*(1), 205-211.

Gibson, G. R., Saavedra, J. M., MacFarlane, S., & MacFarlane, G. T. (1997). Probiotics and intestinal infections. In *Probiotics 2* (pp. 10-39). Springer Netherlands.

Gibson, L., Paba, A., Pes, M., & Scintu, M. F. (2000). Genotypic and phenotypic heterogeneity among lactococci isolated from traditional Pecorino Sardo cheese. *Journal of applied microbiology*, *89*(2), 191-197.

Goepfert, J. M., & Hicks, R. U. T. H. A. N. N. (1969). Effect of volatile fatty acids on Salmonella typhimurium. *Journal of bacteriology*, *97*(2), 956.

Golnazarian, C. A., DONNELLY, C. W., PINTAURO, S. J., & HOWARD, D. B. (1989). Comparison of infectious dose of Listeria monocytogenes F5817 as determined for normal versus compromised C57B1/6J mice. *Journal of food protection*, *52*(10), 696-701.

Grande, M. J., Lopez, R. L., Abriouel, H., Valdivia, E., Ben Omar, N., Maqueda, M., ... & Galvez, A. (2007). Treatment of vegetable sauces with enterocin AS-48 alone or in combination with phenolic compounds to inhibit proliferation of Staphylococcus aureus. *Journal of Food Protection*, *70*(2), 405-411.

Guarner, F., & Schaafsma, G. J. (1998). Probiotics. *International journal of food microbiology*, *39*(3), 237-238.

Haines, W. C., & Harmon, L. G. (1973). Effect of selected lactic acid bacteria on growth of Staphylococcus aureus and production of enterotoxin. *Applied microbiology*, *25*(3), 436-441.

Hardie, J. M., & Whiley, R. A. (1994). Recent developments in streptococcal taxonomy: their relation to infections. *Reviews in Medical Microbiology*, *5*(3), 151-162.

Hardie, J. M., & Whiley, R. A. (1995). The genus Streptococcus. In *The genera of lactic acid bacteria* (pp. 55-124). Springer US.

Hawes, S. E., Hillier, S. L., Benedetti, J., Stevens, C. E., Koutsky, L. A., Wølner-Hanssen, P., & Holmes, K. K. (1996). Hydrogen peroxide—producing lactobacilli and acquisition of vaginal infections. *Journal of Infectious Diseases*, *174*(5), 1058-1063.

Hayes, M., Coakley, M., O'sullivan, L., & Stanton, C. (2006). Cheese as a delivery vehicle for probiotics and biogenic substances. *Australian Journal of Dairy Technology*, *61*(2), 132.

Heng, N. C., Wescombe, P. A., Burton, J. P., Jack, R. W., & Tagg, J. R. (2007). The diversity of bacteriocins in Gram-positive bacteria. In *Bacteriocins* (pp. 45-92). Springer Berlin Heidelberg.

Henning, C., Vijayakumar, P., Adhikari, R., Jagannathan, B., Gautam, D., & Muriana, P. M. (2015). Isolation and taxonomic identity of bacteriocin-producing lactic acid bacteria from retail foods and animal sources. *Microorganisms*, *3*(1), 80-93.

Hillier, S. L., Krohn, M. A., Klebanoff, S. J., & Eschenbach, D. A. (1992). The relationship of hydrogen peroxide-producing lactobacilli to bacterial vaginosis and genital microflora in pregnant women. *Obstetrics & Gynecology*, *79*(3), 369-373.

Hirsch, A., Grinsted, E., Chapman, H. R., & Mattick, A. T. R. (1951). 446. A note on the inhibition of an anaerobic sporeformer in Swiss-type cheese by a nisin-producing streptococcus. *Journal of Dairy Research*, *18*(2), 205-206.

Hoover, D. G., & Steenson, L. R. (Eds.). (2014). Bacteriocins of lactic acid bacteria. Academic Press.

Hviid, A. M. M., Ruhdal-Jensen, P., & Kilstrup, M. (2017). Butanol is cytotoxic to Lactococcus lactis while ethanol and hexanol are cytostatic. *Microbiology*, *163*(4), 453-461.

Jacobsen, T., Budde, B. B., & Koch, A. G. (2003). Application of Leuconostoc carnosum for biopreservation of cooked meat products. *Journal of Applied Microbiology*, *95*(2), 242-249.
JACQUES, N. A., HARDY, L., KNOX, K. W., & WICKEN, A. J. (1980). Effect of Tween 80 on the morphology and physiology of Lactobacillus salivarius strain IV CL-37 grown in a chemostat under glucose limitation. *Microbiology*, *119*(1), 195-201.

Johnson-Henry, K. C., Mitchell, D. J., Avitzur, Y., Galindo-Mata, E., Jones, N. L., & Sherman, P. M. (2004). Probiotics reduce bacterial colonization and gastric inflammation in H. pylori-infected mice. *Digestive diseases and sciences*, *49*(7), 1095-1102.

Jung, D. S., Bodyfelt, F. W., & Daeschel, M. A. (1992). Influence of Fat and Emulsifiers on the Efficacy of Nisin in Inhibiting Listeria monocytogenes in Fluid Milk1. *Journal of Dairy*

Science, *75*(2), 387-393.

Keymanesh, K., Soltani, S., & Sardari, S. (2009). Application of antimicrobial peptides in agriculture and food industry. *World Journal of Microbiology and Biotechnology*, *25*(6), 933-944.

Kim, D. S., Thomas, S., & Fogler, H. S. (2000). Effects of pH and trace minerals on long-term starvation of Leuconostoc mesenteroides. *Applied and environmental microbiology*, *66*(3), 976-981.

Kim, M. H., Kong, Y. J., Baek, H., & Hyun, H. H. (2006). Optimization of culture conditions and medium composition for the production of micrococcin GO5 by Micrococcus sp. GO5. *Journal of biotechnology*, *121*(1), 54-61.

Klaenhammer, T. R. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS microbiology reviews*, *12*(1-3), 39-85.

Klaenhammer, T.R. (1988) Bacteriocins of lactic acid bacteria. Btochimie 70,337-349.
Kniel, K. E., Montville, T. J., & Matthews, K. R. (2012). Food Microbiology: An Introduction (3rd Edition). ASM Press

Kong, S., & Davison, A. J. (1980). The role of interactions between O2, H2O2,· OH, e− and O2− in free radical damage to biological systems. *Archives of biochemistry and biophysics*, *204*(1), 18-29.

König, H., & Fröhlich, J. (2017). Lactic acid bacteria. In *Biology of Microorganisms on Grapes, in Must and in Wine* (pp. 3-41). Springer, Cham.

Kouakou, P., Ghalfi, H., Dortu, C., Evrard, P., & Thonart, P. (2010). Combined use of bacteriocin-producing strains to control Listeria monocytogenes regrowth in raw pork meat. *International journal of food science & technology*, *45*(5), 937-943.

Lyon, W. J., & Glatz, B. A. (1993). Isolation and purification of propionicin PLG-1, a bacteriocin produced by a strain of Propionibacterium thoenii. *Applied and environmental microbiology*, *59*(1), 83-88.

Macwana, S. J., & Muriana, P. M. (2012). A 'bacteriocin PCR array'for identification of bacteriocin-related structural genes in lactic acid bacteria. *Journal of microbiological methods*, 88(2), 197-204.

Malheiros, P. S., Sant'Anna, V., Todorov, S. D., & Franco, B. D. (2015). Optimization of growth and bacteriocin production by Lactobacillus sakei subsp. sakei2a. *Brazilian Journal of Microbiology*, *46*(3), 825-834.

Martinez, F. A. C., Domínguez, J. M., Converti, A., & de Souza Oliveira, R. P. (2015). Production of bacteriocin-like inhibitory substance by Bifidobacterium lactis in skim milk supplemented with additives. *Journal of Dairy Research*, *82*(3), 350-355.

Martínez-Viedma, P., Abriouel, H., Omar, N. B., Valdivia, E., López, R. L., & Gálvez, A. (2008). Inactivation of exopolysaccharide and 3-hydroxypropionaldehyde-producing lactic acid bacteria in apple juice and apple cider by enterocin AS-48. *Food and chemical toxicology*, *46*(3), 1143-1151.

Martley, F. G., & Crow, V. L. (1993). Interactions between non-starter microorganisms during cheese manufacture and repening. *International Dairy Journal*, *3*(4-6), 461-483.

Mathara, J. M., Schillinger, U., Guigas, C., Franz, C., Kutima, P. M., Mbugua, S. K., ... & Holzapfel, W. H. (2008). Functional characteristics of Lactobacillus spp. from traditional Maasai fermented milk products in Kenya. *International journal of food microbiology*, *126*(1), 57-64.

Matsusaki, H., Endo, N., Sonomoto, K., & Ishizaki, A. (1996). Lantibiotic nisin Z fermentative production by Lactococcus lactis IO-1: relationship between production of the lantibiotic and lactate and cell growth. *Applied Microbiology and Biotechnology*, *45*(1), 36-40.

Mendes-Soares, H., Suzuki, H., Hickey, R. J., & Forney, L. J. (2014). Comparative functional genomics of Lactobacillus spp. reveals possible mechanisms for specialization of vaginal lactobacilli to their environment. *Journal of bacteriology*, *196*(7), 1458-1470.

Midolo, P. D., Lambert, J. R., Hull, R., Luo, F., & Grayson, M. L. (1995). In vitro inhibition of Helicobacter pylori NCTC 11637 by organic acids and lactic acid bacteria. *Journal of Applied Bacteriology*, *79*(4), 475-479.

Molinos, A. C., Abriouel, H., López, R. L., Omar, N. B., Valdivia, E., & Gálvez, A. (2008). Inhibition of Bacillus cereus and Bacillus weihenstephanensis in raw vegetables by application of washing solutions containing enterocin AS-48 alone and in combination with other antimicrobials. *Food microbiology*, *25*(6), 762-770.

MORRIS, B. J., IWAMOTO, H. S., & REID, I. A. (1979). Localization of angiotensinogen in rat liver by immunocytochemistry. *Endocrinology*, *105*(3), 796-800.

Mørtvedt, C. I., Nissen-Meyer, J., Sletten, K., & Nes, I. F. (1991). Purification and amino acid sequence of lactocin S, a bacteriocin produced by Lactobacillus sake L45. *Applied and environmental microbiology*, *57*(6), 1829-1834.

Mortvedt-Abildgaa, C. I., Nissen-Meyer, J., Jelle, B., Grenov, B., Skaugen, M., & Nes, I. F. (1995). Production and pH-Dependent Bactericidal Activity of Lactocin S, a Lantibiotic from Lactobacillus sake L45. *Applied and Environmental Microbiology*, *61*(1), 175-179.

Müller-Auffermann, K., Grijalva, F., Jacob, F., & Hutzler, M. (2015). Nisin-producing microorganisms and their implementation in brewers' wort. *Journal of the Institute of Brewing*, *121*(3), 320-331.

Muriana, P. M., & Klaenhammer, T. R. (1987). Conjugal transfer of plasmid-encoded determinants for bacteriocin production and immunity in Lactobacillus acidophilus 88. *Applied and Environmental Microbiology*, *53*(3), 553-560.

Murphy, M. G., & Condon, S. (1984). Correlation of oxygen utilization and hydrogen peroxide accumulation with oxygen induced enzymes in Lactobacillus plantarum cultures. *Archives of microbiology*, *138*(1), 44-48.

Naidu, A. S., Bidlack, W. R., & Clemens, R. A. (1999). Probiotic spectra of lactic acid bacteria (LAB). *Critical reviews in food science and nutrition*, *39*(1), 13-126.

Naveena, B. J., Altaf, M., Bhadriah, K., & Reddy, G. (2005). Selection of medium components by Plackett–Burman design for production of L (+) lactic acid by Lactobacillus amylophilus GV6 in SSF using wheat bran. *Bioresource technology*, *96*(4), 485-490.

Nesbakken, T., Kapperud, G., & Caugant, D. A. (1996). Pathways of Listeria monocytogenes contamination in the meat processing industry. *International Journal of Food Microbiology*, *31*(1-3), 161-171.

Nissen-Meyer, J., & Nes, I. F. (1997). Ribosomally synthesized antimicrobial peptides: their function, structure, biogenesis, and mechanism of action. *Archives of microbiology*, *167*(2), 67-77.

Parente, E., Ricciardi, A., & Addario, G. (1994). Influence of pH on growth and bacteriocin production byLactococcus lactis subsp. lactis 14ONWC during batch fermentation. *Applied Microbiology and Biotechnology*, *41*(4), 388-394.

Patterson, M. J. (1996). Streptococcus.

Piard, J. C., & Desmazeaud, M. (1992). Inhibiting factors produced by lactic acid bacteria. 2. Bacteriocins and other antibacterial substances. *Le lait*, *72*(2), 113-142.

Piard, J. C., Delorme, F., Giraffa, G., Commissaire, J., & Desmazeaud, M. J. (1990). Evidence for a bacteriocin produced by Lactococcus lactis CNRZ 481. *Netherlands Milk and Dairy Journal*, *44*(3-4), 143-158.

Pinto, M. S., de Carvalho, A. F., dos Santos Pires, A. C., Souza, A. A. C., da Silva, P. H. F., Sobral, D., ... & de Lima Santos, A. (2011). The effects of nisin on Staphylococcus aureus count and the physicochemical properties of Traditional Minas Serro cheese. *International dairy*

journal, *21*(2), 90-96.

Prudêncio, C. V., Dos Santos, M. T., & Vanetti, M. C. D. (2015). Strategies for the use of bacteriocins in Gram-negative bacteria: relevance in food microbiology. Journal of food science and technology, 52(9), 5408-5417.

Pulido, R. P., Omar, N. B., Abriouel, H., López, R. L., Cañamero, M. M., & Gálvez, A. (2005). Microbiological study of lactic acid fermentation of caper berries by molecular and culture-dependent methods. *Applied and environmental microbiology*, *71*(12), 7872-7879.

Radha, K. R., & Padmavathi, T. (2017). Statistical optimization of bacteriocin produced from Lactobacillus delbrueckii subsp bulgaricus isolated from yoghurt. *International Food Research Journal*, *24*(2).

Ravi, V., Prabhu, M., & Subramanyam, D. (2017). Isolation of bacteriocin producing bacteria from mango pulp and its antimicrobial activity. *Journal of Microbiology and Biotechnology Research*, *1*(2), 54-63.

Rink, R., Wierenga, J., Kuipers, A., Kluskens, L. D., Driessen, A. J., Kuipers, O. P., & Moll, G. N. (2007). Production of dehydroamino acid-containing peptides by Lactococcus lactis. *Applied and environmental microbiology*, *73*(6), 1792-1796.

Sabia, C., De Niederhäusern, S., Messi, P., Manicardi, G., & Bondi, M. (2003). Bacteriocin-producing Enterococcus casseliflavus IM 416K1, a natural antagonist for control of Listeria monocytogenes in Italian sausages ("cacciatore"). *International Journal of Food Microbiology*, *87*(1), 173-179.

Salminen, S., & Gueimonde, M. (2004). Human studies on probiotics: what is scientifically proven. *Journal of food science*, *69*(5).

Sanders, M. E. (1993). Effect of consumption of lactic cultures on human health. *Advances in food and nutrition research*, *37*, 67-130.

Savadogo, A., Ouattara, A. C., Bassole, H. I., & Traore, S. A. (2006). Bacteriocins and lactic acid bacteria-a minireview. *African journal of biotechnology*, *5*(9).

Shareck, J., Choi, Y., Lee, B., & Miguez, C. B. (2004). Cloning vectors based on cryptic plasmids isolated from lactic acid bacteria: their characteristics and potential applications in biotechnology. *Critical reviews in biotechnology*, *24*(4), 155-208.

SKINNER, F. A., & QUESNEL, L. B. (1978). Streptococci. The Society for Applied Bacteriology Symposium Ser. In *Streptococci. The Society for Applied Bacteriology Symposium Ser.* (No. 7). Academic Press Inc.(London) Ltd. 24-28 Oval Road London NW17DX..

Souza, E. C., Azevedo, P. O. D. S. D., Domínguez, J. M., Converti, A., & Oliveira, R. P. D. S. (2017). Influence of temperature and pH on the production of biosurfactant, bacteriocin and lactic acid by Lactococcus lactis CECT-4434. *CyTA-Journal of Food*, 1-6.

SPELHAUG, S. R., & HARLANDER, S. K. (1989). Inhibition of foodborne bacterial pathogens by bacteriocins from Lactococcus lactis and Pediococcus pentosaceous. *Journal of Food Protection*, *52*(12), 856-862.

Todorov, S. D., & Dicks, L. M. T. (2005). Effect of growth medium on bacteriocin production by Lactobacillus plantarum ST194 BZ, a strain isolated from boza. *Food Technology and Biotechnology*, *43*(2), 165-173.

Todorov, S. D., Wachsman, M., Tomé, E., Dousset, X., Destro, M. T., Dicks, L. M. T., ... & Drider, D. (2010). Characterisation of an antiviral pediocin-like bacteriocin produced by Enterococcus faecium. *Food Microbiology*, *27*(7), 869-879.

Todorov, S., Onno, B., Sorokine, O., Chobert, J. M., Ivanova, I., & Dousset, X. (1999). Detection and characterization of a novel antibacterial substance produced by Lactobacillus plantarum ST 31 isolated from sourdough. *International Journal of Food Microbiology*, *48*(3), 167-177.

Turgis, M., Vu, K. D., Millette, M., Dupont, C., & Lacroix, M. (2016). Influence of environmental factors on bacteriocin production by human isolates of Lactococcus lactis MM19 and Pediococcus acidilactici MM33. *Probiotics and antimicrobial proteins*, *8*(1), 53-59.

Vignolo, G. M., Kairuz, M. N., Holgado, A. A., & Oliver, G. (1995). Influence of growth conditions on the production of lactocin 705, a bacteriocin produced by Lactobacillus casei CRL 705. *Journal of Applied Microbiology*, *78*(1), 5-10.

Vliegen, I., Jacobs, J. A., Beuken, E., Bruggeman, C. A., & Vink, C. (2006). Rapid identification of bacteria by real-time amplification and sequencing of the 16S rRNA gene. *Journal of microbiological methods*, *66*(1), 156-164.

Walker, S. J., Archer, P., & Banks, J. G. (1990). Growth of Listeria monocytogenes at refrigeration temperatures. *Journal of applied microbiology*, *68*(2), 157-162.

Whiley, R. A., Beighton, D., Winstanley, T. G., Fraser, H. Y., & Hardie, J. M. (1992). Streptococcus intermedius, Streptococcus constellatus, and Streptococcus anginosus (the Streptococcus milleri group): association with different body sites and clinical infections. *Journal of clinical microbiology*, *30*(1), 243-244.

Yamauchi, Y., Ishii, S., Toyoda, S., & Ahiko, K. (1996). *U.S. Patent No. 5,527,505*. Washington,

DC: U.S. Patent and Trademark Office.

Yang, E., Fan, L., Jiang, Y., Doucette, C., & Fillmore, S. (2012). Antimicrobial activity of bacteriocin-producing lactic acid bacteria isolated from cheeses and yogurts. *AMB Express*, *2*(1), 48.

Zottola, E. A. (1994). Food Micro'93—15th International Symposium: the International Committee on Food Microbiology and Hygiene: Bingen, Germany, 31 August–3 September 1993. *Food Control*, *5*(1), 49.